

When is genetic screening permissible?

The World Medical Association is planning to fashion a statement on the ethical principles of genetic screening from a draft that is altogether over-cautious.

THAT there should be general anxiety about the implications of the Human Genome Project (in its many national manifestations) is hardly surprising: the technology is new and will eventually illuminate the physical and even the behavioural constitution of individuals. The *Genome Directory* published as a supplement to this issue of *Nature* is a vivid illustration both of how much has been accomplished in the past few years and of how much remains to be done before the databanks contain a complete and representative nucleotide sequence of the human genome. But even when that is done, the task will remain of understanding both the functions of all the genes that will be revealed and the manner in which they are regulated by signals from within and external to the cells containing them. The project is unlikely ever to be complete in a tidy sense, but it will dominate the agenda for research in genetics for decades to come.

For just these reasons, social responses to deepening understanding of genetics cannot be tidy either. Already, and properly, there has been vigorous discussion of the use of genetic screening in the contexts of personal insurance and employment. Fears abound that genetic information will be used unfairly to discriminate against members of a population who are already cruelly disadvantaged by their genetic constitutions. To the extent that such a thing is possible, a consensus is emerging that genetic information should be confidential to individuals and their physicians (begging the question of insurance companies' standard requirement that would-be policy-holders should disclose all relevant information at the outset).

Privacy is the cornerstone of the World Medical Association's draft statement on genetic diagnosis (see page 279), which the association hopes will be accepted, possibly after amendment, during 1996. Amendment there should certainly be, for the present draft (devised by the association's Belgian constituent) is needlessly cautious, in some ways too restrictive and in others plainly unrealistic. Even the principal recommendation, that physicians should not embark on genetic diagnosis without their patients' consent, is disputable in the now-novel circumstances; should a physician be hampered in providing the best care for a patient genetically susceptible to heart disease by the patient's wish not to know the genetic facts of his or her life? A patient's privacy is paramount, but not within his or her relationship with the physician.

A proposed rider to this restriction would be even more irksome. The draft statement says that genetic diagnosis

should be carried out only when there is a therapeutic or prophylactic remedy for the genetic condition identified, or when its outcome will inform "reproductive decisions". Why not also accede to patients' wish to know their status in respect to a genetically determined condition? Thus a person with late-onset Alzheimer's disease in the family would no doubt be relieved to know that he or she did not have two copies of allele-4 of the APO-E (apolipoprotein-E) gene, and otherwise would be encouraged to keep personal affairs in good order.

The underlying and false assumption is that patients should never have thrust upon them potentially disquieting information. That there should be perceptive counselling and follow-up when such information has to be communicated goes without saying (and is said often in the draft), but the drafters have underestimated the degree of public knowledge of genetics recently spread (often, sadly, in the form of over-sharp genetic determinism). They have also failed to allow for the likelihood that only when there is a wider knowledge of individuals' genetic predisposition to conditions now supposed to be intractable, such as Alzheimer's disease, will it be possible to identify life-style factors that may yet prove to be prophylactic for these conditions. In the longer run, wider genetic self-awareness will help bring about the more rational attitude towards the inevitability of death of which modern societies (and health-care systems) are in great need.

But the oddest feature of the World Medical Association's document is not a recommendation as to how physicians should behave, but part of the preamble where it is stated that prophylactic measures (to counteract the effects of deleterious genes) may lead to the survival of people "presenting altered genes" and may thus "impede the procedures of natural selection". What, one is bound to ask, has modern medicine been up to all these years? In recent decades, antibiotics have probably done more than the prophylaxis of genetically determined conditions ever will artificially to increase people's Darwinian fitness. The techniques of pre-natal genetic diagnosis followed by abortion will go much further to repeal the Hardy-Weinberg law as it might apply to the balance of human genes. That practice can only grow as the repertoire of genes identifiable *in utero* is enlarged. If proper care is taken (on such matters as the potential of unexpected heterozygous advantage, for example), the result should be a fitter population (in the non-Darwinian sense). Is that not why physicians are in business? □



More central control

British universities, which award degrees by examining their students, are now to be examined themselves.

THE British government's hankering for central control is evidently boundless. So much is evident in the decision by the Department for Education last week to set up a new organization to superintend educational standards in British universities. The idea is that, beginning in 1997, the new organization should carry out biennial inspections of university institutions. One of the objectives is said to be that the organization will ensure that degrees awarded by different institutions are of a "broadly comparable standard". The government's statement also says that the aim is to ensure "accountability for public funds" and respect for "university autonomy over academic standards".

Administratively, the new arrangement makes sense. Hitherto, responsibility for standards in higher education has been dispersed; the universities themselves have run the Higher Education Quality Council, while each of the four funding councils that allocate funds to different institutions rely on their own assessments in making their decisions. Universities will have only one invigilator breathing down their necks, which is no doubt why the chairman of the Committee of Vice-Chancellors and Principals described himself as "absolutely delighted" with the new arrangements. But he, and the universities, should think again.

Since the snap decision in 1990 that former polytechnics should be allowed to call themselves universities, the basis of the British government's relationship with universities has been the market principle that institutions must stand on their own feet, competing in the market for research funds and for students. The logic of that position is that there will in future be a variety of universities, offering courses with varied content and of varied difficulty to students of different aptitudes. Socially, that would be a prize worth having. Any system of higher education in which the participation rate approaches 30 per cent needs a substantial complement of second-rate universities — and, crucially, incentives by which those institutions will seek better things. The doctrine that "a degree is a degree is a degree...", for which British industry and politicians have been baying in recent months, is inimical to the diversity the British system needs. □

Technophobia and shares

The London Stock Exchange, which thinks itself a citadel of capitalism, is behaving badly towards the Internet.

HERE is a fable of our times. Two smallish and technically alert British companies decided that it would be a good idea to use the Internet for selling shares in publicly registered British companies. One of them, called Sharelink, is a regular stockbroker that specializes in selling shares in small numbers to small investors, the other is a small Internet ser-

vice provider based at Cambridge. The technical difficulties are not great and were quickly mastered, but the consortium needed a source of prices at which it would offer to buy or sell particular shares. Sharelink, in its capacity as a stockbroker, has access to the London Stock Exchange's electronic stream of trading prices, but properly recognized that it could not relay them to the Internet without breaching the exchange's copyright. So, for an interim period, the two sides made a deal: the exchange would be paid one penny (£0.01) for each item of its copyright information passed on to a potential buyer or seller of stock. Knowing that the secret of the Internet, that it is one thing to have a 'site' and quite another to know whether anybody will 'visit' it, the consortium arranged (two weeks ago) for a great party at which their venture would be publicized. And then the skies fell in.

To everybody's astonishment, the London Stock Exchange, which likes to think of itself as the world's most influential market in securities, repudiated its contract with the untried enterprise. When an official of Sharelink told a radio reporter that he considered the exchange's action unfair (or words to that effect), the exchange threatened to sue him for defamation. Goliath, it seemed, had been rattled by the mere appearance of an untried David on the scene, its own well-known distaste for small trades in stocks notwithstanding. But against a mounting background of ridicule, the exchange had second thoughts. In a weekend meeting ten days ago, the consortium and the exchange hammered out a new agreement; the use of the Internet for buying and selling shares will be allowed. So have reason and virtue triumphed over anti-competitive hankerings?

Not a bit of it. Another row, centred on an operation called Tradepoint, has arisen to take the place of the Sharelink scheme. That venture seeks to deal in very large, not very small, parcels of shares; its clients would be pension funds and other financial institutions. Its rationale is that, because the cost of dealing in large blocks of shares may be less than that of dealing in small bundles, it should be able to offer large institutions better prices than those obtainable from other stockbrokers. But that would offend against rule 4.18 of the 'rule book' with which all corporate members of the Stock Exchange must comply, that they do not offer more favourable prices than those offered by the Stock Exchange's own electronic datastream. Now the big, not the small, moneybags are up in arms.

Like other stock exchanges, the London Stock Exchange is a consortium of its stockbroker members; it seeks to protect their interests and to make a profit. Its almost instinctive reaction against both Sharelink and Tradepoint reflects the fear of most member firms that both initiatives will take business from them. But why was not the exchange itself, acting on behalf of its members, the first with the innovations it has been trying to squash? Sadly, this is the same stock exchange that, a year ago, lost close on £100 million of its members' money in devising a computer system to handle the accounting for stock sales. Lapsing into technophobia is understandable in the circumstances, but is hardly encouraging for those who pin their faith on the exchange's capacity to keep its preeminent position in a technical world. □

EC plans encryption rules in bid to police information superhighway

Paris. The European Commission is to propose legislation to police the information superhighway that will include powers to decrypt confidential telephone and computer communication.

The commission's move follows concern over the perceived increase in the 'illegal' use of the Internet, including the proliferation of pornography and the unauthorized release of classified documents.

It also coincides with a similar proposal from the 34-nation-member Council of Europe. The proposals would, if passed into law, effectively end the Internet's status in the 15 member states of the European Union (EU) as an unregulated medium for the free flow of information.

But they have also raised questions about the possible violation of telephone and computer privacy, as well as the preferred choice of encryption/decryption system.

The proposal to introduce Europe-wide surveillance guidelines has been confirmed by a senior official responsible for encryption and data security in the French government. He says that Brussels is working closely with the Senior Officers' Group for Information Security Systems (SOGIS), a collection of experts from EU countries, chaired by the commission itself.

The commission is expected to publish its guidelines later this autumn, detailing the powers of enforcement to be given to regulatory authorities, as well as a preferred choice of decryption system. The guidelines will then be considered by the EU's Council of Ministers and the European Parliament.

But a spokesman for the commission's

telecommunications directorate says that the decryption mechanism is likely to be based on a version of the 'key escrow system'. This refers to the policy under which users of encryption systems give copies of their decryption keys either to their government or to a third party that the government trusts. The keys can be handed over if the government, on production of a court order, wants to monitor encrypted information.

The system being considered by the commission will enable EU countries to monitor encrypted telephone and computer communications in member states. Thus if someone in Germany makes a call to Italy, agencies in both countries would possess the keys enabling them to decrypt the call.

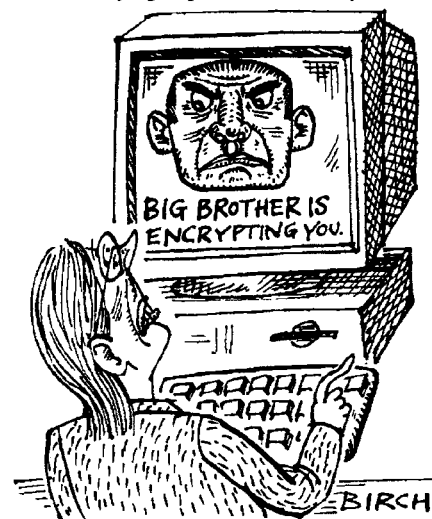
Significantly, the commission will also propose that member states choose private 'trusted third parties' rather than government departments to regulate computer and telephone networks. It is thought to believe that this move will secure greater public support for the proposals.

But civil liberties groups remain sceptical, and maintain that the use of 'third parties' to police the Internet raises its own questions, one of which is deciding which party to trust and ensuring they all remain trustworthy. "It is difficult to trust these third parties," says Simon Davies from the organization Privacy International. "There is no guarantee that the keys [to decryption] will not be corruptly accessed within the 'trusted' organization."

Critics of the commission's proposals also include information technology specialists, although their concerns are different. Ross Anderson, a senior research associate in

computer and communications security at the University of Cambridge's Computer Laboratory says that the Council of Ministers will need to iron out various issues before the key escrow system is fit for use.

One factor, says Anderson, is that such a system ironically falls victim to precisely what it is trying to protect — namely, nation-



al security. "If you are a banker doing a politically sensitive deal — such as renegotiating the Eurotunnel debt — then the UK government will certainly not want the French to get that key."

Similarly, the decryption key for a secure telephone bought in Britain will be kept at the government's General Communications Headquarters. But if it is taken into France and used to call someone in Germany, the French government "will inevitably want a copy of the key", says Anderson.

This direct conflict of national security priorities, adds Anderson, makes it hard to "specify a system which satisfies the curiosity of intelligence agencies, while still providing meaningful privacy to users".

A parallel proposal for decryption was announced earlier this month by the Council of Europe. Peter Csonka, head of the council's Crime Problems Division, said its 18 suggestions were long overdue following concern that "electronic information systems and electronic information may also be used for committing criminal offences".

The council's suggestions include giving investigating agencies the right to search computer networks and seize offending, unauthorized or illegal material. The proposals will also require providers of telecommunication networks to "avail themselves of all necessary technical measures that enable the interception of telecommunications by investigating authorities". **Jerome Thorel**

Royal Society opens rebuilt lecture hall

London. **Britain's Royal Society last week re-opened its 168-year-old lecture theatre (right), newly refurbished after asbestos was discovered in the ceiling in 1994. It is now named the Wellcome Trust Lecture Hall.**

The new facilities, according to a Royal Society spokeswoman, "blend the prestigious and elegant atmosphere of Carlton House Terrace, designed in 1827, with modern comfort and state-of-the-art technology".

The Royal Society itself was founded in 1660, by a group of 12 founding



fellows including Robert Boyle. The lecture hall was opened on Tuesday with a quiz pitting a team from Trinity College, Cambridge, this year's winners of the television quiz show *University Challenge*, against a one made up of fellows of the society. □

Crime and genetics conference breeds further controversy

Washington. A long-delayed conference on genetics and crime, which finally took place in rural Maryland last weekend, was punctuated by onslaughts from protesters trying to shut it down, and dominated by semantic arguments over words such as 'crime'.

As a result, many participants left the meeting feeling frustrated that it had provided little basis for a substantive debate on the social implications of research in behavioural genetics.

The conference was originally due to have taken place three years ago, but was cancelled when funding from the National Institutes of Health was withdrawn following opposition from the Congressional Black Caucus and others.

Its goal was to provide a forum for discussing both the scientific data and the political implications of work in this field. The organizer of the conference, David Wasserman of the University of Maryland, says that its primary goal was to explore "the social, legal and ethical implications of the current research on genetics and criminal behaviour".

Irving Gottesman of the University of Virginia — an active researcher in twin studies used to detect the genetic basis for human behaviour — tried to set a conciliatory tone for the meeting at the outset by stating that "everyone at the conference could agree that we must fight genetic discrimination".

But the depth of any such common feeling was repeatedly questioned as 35 scientists, criminologists, historians and philosophers each took the floor during the three-day event, held at the Aspen Institute in Queenstown, near Annapolis.

Several researchers insisted that their research was non-racist, for example where it was limited to studies of Caucasians in countries such as Denmark with little racial conflict. But Kathryn Russell, of the University of Maryland's department of criminal justice, said that the implications of even this type of research were "inherently racist", as it was based on a racially biased view of crime.

Indeed, semantics emerged as the dominant theme of the meeting, with lengthy arguments about the meaning and interpretation of words such as 'heritability' and even 'biological'.

The focus on such topics, rather than on behavioural research and its potential dangers, led some participants to question whether the discussions were moving in any productive direction, while expressing frustration at the brevity of the scientific presentations, and inadequate time for questions and answers. Laurie Goodman

Budget kills off US biological service and cuts research

Washington. The two-year saga of the US National Biological Service (NBS), the controversial agency created by the Interior Secretary, Bruce Babbitt, to focus biological research within the federal government, came to an abrupt end last week.

The House of Representatives and the Senate agreed a budget for the Department of the Interior that abolishes the NBS and transfers its functions to the US Geological Survey (USGS). Funds allotted to "natural resources research" within the USGS next year will be \$137 million — \$9 million less than the amount proposed by the Senate for the NBS, but \$25 million more than the figure in the House, where legislators had proposed a merger with USGS earlier this year.

The White House immediately threatened to veto the Interior appropriations bill over items such as logging in Alaska and the Bureau of Indian Affairs. But the NBS is unlikely to figure in any last-minute negotiations between the Congress and the president. Babbitt's plan for a strong and independent ecological research agency — which is something the NBS never had the chance to become in its short and turbulent life — therefore appears to have failed.

Exactly how the USGS will absorb the former agency's functions is still to be worked out. The House-Senate committee that made last week's decision instructed the USGS to start dismantling and shrinking the NBS from 1 October, and to report back within nine months on how natural resources research will be integrated into the survey's activities.

Some NBS employees, most of whom were moved from the Fish and Wildlife Service when the new agency was created in

1993, are certain to lose their jobs, as next year's funding level is about 13 per cent lower than this year's. Congress ordered reductions to be made "predominately in administrative, managerial, and other headquarters support functions".

Whether any of the existing NBS centres will have to close is not certain; the Southeast Science Center in Gainesville, Florida, is considered especially vulnerable. Ronald Pulliam, director of the NBS, admitted last week that some continuing long-term studies will have to be scrapped, as will new strategic research.

Indeed, the report accompanying the Interior Department's appropriations bill spells out what kind of "natural resources research" Congress expects the USGS to conduct with its new-found money. The research, it says, should be "closely linked to management issues", implying that it should stick to the kind of work the Fish and Wildlife Service had been doing before NBS was created, such as keeping track of fish and game stocks.

Conservative members of Congress had been sceptical about the NBS from its inception, fearing any wide-ranging ecological surveys would result in more private property being placed off limits to developers.

But one congressional threat to long-term ecological research was removed last week. A Senate amendment prohibiting aerial surveys without written consent of property owners (see *Nature* 376, 627; 1995) was changed to apply only to surveys "for the designation of habitat under the Endangered Species Act". This should allow work using remote-sensing data to map biological resources to continue. **Tony Reichhardt**

NASA prepares for antimatter experiment

Washington. The US Department of Energy has signed an agreement with the National Aeronautics and Space Administration (NASA) to fly an experiment designed to detect antimatter on the space shuttle and the international space station.

The experiment team for the Alpha Magnetic Spectrometer (AMS), which is made up of scientists from 37 universities and laboratories, is led by Samuel Ting of the Massachusetts Institute of Technology, a joint winner of the 1976 Nobel Prize in physics.

The agreement marks the first time another government agency has sponsored a major experiment on the space station. Daniel Goldin, the NASA Administrator, wasted no time in boasting about Ting's involvement. "I've always said that the space station will be an orbiting laboratory capable

of conducting world-class science, and the addition of an experiment whose science team is led by a Nobel laureate is one more step in realizing its full potential."

The heart of the AMS is a cylindrical magnet, one metre in length and one metre in diameter, through which high-energy antimatter particles would pass. The agreement calls for the device to be tested on shuttle mission STS-90 in April 1998, and then to be placed on the space station in 2001 for three years. The project involves collaborators from several countries, including Italy, Switzerland, Germany and China.

DoE will pay \$3 million for the experiment. NASA is providing \$12.7 million towards its costs, primarily to pay for integrating the payload into the shuttle and space station. **T. R.**

Spermatid injection fertilizes ethics debate

Paris. A fierce debate has reopened in France over the ethics of creating children by directly injecting human eggs with individual spermatozoa — a technique known as intracytoplasmic sperm injection (ICSI).

The controversy follows the extension of the technique to the injection of spermatids, the round unflagellated precursor cells of spermatozoa that are normally only found in the testicles but also occur in the semen of many infertile men who lack spermatozoa — a condition known as azoospermia.

At the heart of the dispute is concern that the treatment is already being used on experimental human subjects without what critics consider to be either adequate prior animal studies or evidence that the condition is sufficiently serious to justify such studies being bypassed.

Earlier this year, spermatids from sterile males were injected into human eggs in an attempt to remedy azoospermia in seven test couples. The first baby produced using the technique was born in June, and the second last week, at the American Hospital in Paris. No genetic or morphological abnormalities have been detected in either babies.

The results were reported in August in the *New England Journal of Medicine* by Jacques Testart, head of the Gamete Maturity and Fertilization Laboratory in Paris of INSERM, the national biomedical research organization, working in collaboration with Jan Tesarik, of the American Hospital in Paris, and Carmen Mendoza of the University of Granada in Spain.

Ironically, Testart is one of France's most outspoken critics of the ethical risks of medically assisted procreation. In 1982, he became one of the 'fathers' of France's first test-tube baby, Amandine. Four years later, however, he called for a moratorium on such research, in particular because of his concern that the techniques could be used for eugenic ends, and announced that he himself was giving up work on *in vitro* fertilization (IVF) — apart from its use to treat infertility (see *Nature* 385, 323; 1986).

Now Testart is under attack over what Axel Kahn, director of INSERM's genetics and molecular pathology research laboratory at the Cochin Institute of Molecular Genetics in Paris, calls "the biggest ethical problem since the development of medically assisted procreation".

In particular, Kahn claims that Testart's experiments — as indeed other human tests of ICSI — conflict with the 1947 Nuremberg code of medical ethics. This states that experiments on humans should be preceded by adequate animal experiments, and that any risks taken should correspond to the urgency of a given situation.

Kahn claims that ICSI is aimed at treating sterile couples who already have the alternative of using sperm from anonymous

donors. He argues that while using gametes from the male partner might be considered preferable to those from an anonymous donor, the medical condition of such couples is not sufficiently urgent to justify the use of highly experimental techniques.

He also points out that the original ICSI technique was developed without any preliminary experiments on animals.

Testart has replied that he tested his technique in rabbits and mice, leading to the birth of healthy young which themselves were able to reproduce. But Kahn claims that the animals used were not appropriate models, as they were fertile and produced normal healthy sperm.

Speaking at the XV World Congress on Fertility and Sterility at Montpellier last week, Testart rebutted such criticisms. "What sort of discovery or scientific innovation can we expect which would allow us to act with complete certainty as to the results? None!" he said. "We're in a period — as often in medicine — where the choice is between doing nothing or doing something."

Testart also claims that the evolution of the spermatid into a spermatozoon does not appear to correspond to any modification necessary to embryonic development, but only to the acquisition of the properties needed for the transport of the male gamete and its fusion with the egg.

The risks involved, he has argued, can be "reasonably estimated" as "low or zero", and would in any case affect only the early viability of the zygote, not the fetus or child. Spermatids are genetically mature, he claims, adding that it is "difficult to imagine how their contribution to the embryonic genome could lead to abnormalities".

But critics such as Kahn argue that there are legitimate reasons for concern that ICSI — in particular using spermatids — might be dangerous, in particular because the use

of cells selected at random avoids the competition between sperm that takes place in conventional IVF. Geneticists at last week's meeting in Montpellier also argued that sterility may result from serious chromosomal accidents, and that artificial means of overcoming such sterility may therefore risk producing seriously deformed children.

Indeed, the French national bioethics consultative committee last year advised that ICSI should be carried out only after comprehensive animal experiments have been done. But Testart says that animal experiments could delay therapeutic applications, while not providing any additional useful information.

It is too soon to know the real risks of ICSI. About 600 normal children have been born following conventional ICSI, using spermatozoa. But a group of Belgian and Dutch researchers reported last week, in a paper published in *The Lancet* (773, 346; 1995), that in a study of 15 embryos conceived using ICSI, five showed serious genetic abnormalities in sex chromosomes.

Yet Kahn argues that the outcome of the scientific debate is irrelevant. "Even if ICSI works, it doesn't change my analysis by one iota," he says. "For the first time since the Nuremberg code was drafted, we have embarked on human experiments aimed at creating humans without any great urgency for such experiments."

But Testart argues that the couples prefer the new techniques to using sperm from anonymous donors, and are willing to accept the risk, for example, that the male children born may themselves be sterile, arguing that the parents appreciate that treatments for sterility are likely to become available in their children's lifetime.

Ironically, during the debate over France's bioethics bills in 1993, Testart was among those who called for a total ban on pre-implantation diagnosis and selection of embryos. In line with many researchers and physicians, the National Assembly eventually decided that such techniques should be permitted in "exceptional cases", namely where parents risked bearing a child with a "particularly serious" genetic disorder.

Declan Butler

AZT combination trial shows positive results

London. A trial studying the effects of the anti-AIDS drug AZT when given jointly with one of two antiretroviral drugs has shown that patients with HIV and AIDS receiving the combination therapy survived considerably longer than those receiving AZT alone. As a consequence, the trial, backed by three major pharmaceutical companies, has been stopped early.

The decision to release the preliminary results of the so-called Delta trial, which

started in 1992 and involved more than 3,000 participants in Europe and Australia, was announced earlier this week at a conference in Copenhagen.

The preliminary results showed that 17 per cent of participants who took AZT alone and had not taken the drug before died during the trial, compared with 10 per cent of those who took AZT with didanosine (ddI) and 12 per cent of those who took it with zalcitabine (ddC).

Forensic team digs up Haiti's deadly past

Port-au-Prince, Haiti. Elisyen and Jean-Pierre Dazme were riding pillion on a motor-cycle past an army barracks on the afternoon of 2 October 1991 when, according to friends and family, they were shot in the back by troops involved in a military coup that had taken place two days earlier.

Last week (20 September), their bodies were exhumed from a graveyard at Gonaïves, one hundred miles north of Port-au-Prince, by forensic anthropologists who say that they intend to apply their science to finding out the truth about the cousins' deaths.

The exhumations were carried out by a team of forensic experts from Argentina, Guatemala and the United States who came to the troubled island to help a Commission on Truth and Justice, established in March by President Jean-Bertrand Aristide, to investigate human rights abuses. It has been estimated that more than 3,000 people died at the hands of the now-disbanded Haitian army and its allies between the 1991 coup and Aristide's return a year ago, which was backed by the United States.

"The commission will not investigate all of these cases," says its president, Françoise Boucard. "It intends to clarify the nature of the repression, and identify the character of the human rights violations." Where possible, the commission is also mandated to identify the individuals responsible, although it will not handle their prosecution.

The forensic team is using a wide range of techniques, refined during 15 years of extensive investigations in Argentina and elsewhere in Latin America, to establish identity and cause of death from skeletal remains.

Luis Fondebrider, an Argentinian member of the team, works quickly and meticulously on the exposed cadaver of Elisyen Dazme. "This technical part is only a small component of our work," he explains later.

The larger part, he says, is piecing together information on the circumstances of death and the location of bodies from local people.

Most of Dazme's burial clothes have rotted away. But the synthetic fabric of his tie and socks survive, the latter containing a full collection of foot bones that will later be



Body of evidence: the exhumation of remains begins.

examined at an improvised laboratory at a hospital in Port-au-Prince.

Family and friends look on, sombre but calm, even as the exhumation is accompanied by the ritualized shrieking and wailing of another funeral nearby. They say that they hope the exercise will lead to justice, and are encouraged in this view by Father Daniel Roussi re, a French Roman Catholic priest and human rights activist, who stayed with the people of Gonaïves through the dark days of the repression.

He says that this exhumation, as well as several others recently carried out, will "prove that there was a massacre of the civilian population" after a demonstration against the coup in Gonaïves on 2 October 1991 in which seven people were killed and 80 injured. Roussi re names an army colonel, now in exile in the United States, as being responsible. Other officers involved

are already being prosecuted in Haiti.

Roussi re says that the exhumation found broken bones, indicating that Dazme had been shot. But the scientists were not able to confirm this before the formal examination of the bones at the hospital. The examination will be carried out by Karen Burns of the University of Georgia, a pioneer of forensic anthropology who trained other team members on previous missions. She points out that fatal bullets to the body do not necessarily fracture bones, thus leaving no firm evidence of the cause of death.

Information from the exhumations will be made public in the report of the Truth Commission, due by December, with information compiled on a computer database from 5,000 complaints of human rights abuses filed so far by people all over Haiti.

But the report is unlikely to contain much forensic information about 'killing fields' or mass graves that some islanders have alleged exist at two Port-au-Prince sites.

One, at Titanyen, is a sprawling area of volcanic ash where bodies of the destitute are dumped, unburied; scientists consider it impractical to identify the victims of summary executions among the scavenged remains.

Furthermore, at Fort Dimanche, the city's recently disused prison where the children of newly arrived squatters play amidst the garbage and mosquito-plagued pools of human waste, there are also said to be too many bodies, buried over too many years.

Indeed, according to Jose Pablo Baraybar, a forensic investigator working for the United Nations (UN) civilian mission to Haiti, 'killing fields' as such may not exist in Haiti. The pattern of executions was sporadic, he says, with bodies often left lying in the streets, like those of the Dazme cousins. The exhumations, he hopes, will provide "a missing link between the testimonies of relatives and other kinds of evidence".

Officials of the UN, which is providing some technical support for the Truth Commission but does not itself have a mandate to investigate pre-1993 human rights violations, privately express reservations about the public conduct of the exhumations. Doubts about Aristide's commitment to justice, as opposed to retribution, may also explain the difficulty the commission has had in raising funds from abroad.

But Dan Salcedo of the human rights programme at the American Association for the Advancement of Science, sponsor of the international forensic team, believes that the mission "is working out well", and will help to build democracy and justice in Haiti.

"As fragile as the judicial system is, it is improving, and technical help will be invaluable to that process" he says. "Until you have physical evidence, there are always people that will say that [abuses] just didn't happen."

Colin Macilwain

Funding squeeze for German universities

Munich. Germany's *L nder* governments agreed last week to back a controversial financing plan proposed by the federal government for Germany's university building programme. This limits spending to DM3.6 billion (US\$2.5 billion) in 1996 — rather than the DM4.6 billion that had been earlier recommended by the *Wissenschaftsrat*, the national science council, as the minimum necessary to meet current demands on higher education.

As a result of the decision, no new buildings will be started next year, and only two-thirds of the research equipment costs recommended for support by the *Wissenschaftsrat* will in fact be funded (see *Nature* 373, 95; 1995).

The *L nder*, which share the cost of the building programme with the federal education and research ministry, rejected an offer from the minister, J rgen R ttgers, to add a further DM80 million to the federal government's share in exchange for their support for an additional cost-saving proposal, namely to charge students interest on loans (see *Nature* 376, 105; 1995).

Instead, both sides agreed to set up a working party to investigate new models of financing the building programme. In particular they will study a proposed leasing plan, under which private investors will cover the initial cost of building, to be paid back by universities through long-term leases on the buildings. □

Physicians prepare guidelines on use of human genome data

London. The World Medical Association (WMA) — which brings together the professional physicians' organizations from 64 countries — has agreed to draw up a set of guidelines covering the medical use of information derived from the sequencing of the human genome.

The WMA's move follows similar proposals already adopted by the International Bar Association, which is in the process of preparing a document covering the legal use of such information.

A draft of the guidelines for doctors was presented last week by the Belgian Medical Association to a council session of the WMA held in Bali, Indonesia. The guidelines would take the form of a statement by the WMA on predictive medicine, and follow an earlier declaration on the Human Genome Project issued by the association in 1992.

Each member organization has agreed to discuss the current draft with its members. The results of these consultations will then be discussed by the WMA next year, with a view to producing an agreed text to act as global guidelines for the medical profession.

"The issues to be covered are becoming more urgent as each month passes," says Nigel Duncan, a spokesman for the WMA.

The statement as proposed by the Belgian doctors includes several tough restrictions. In particular, it says that any genetic diagnosis for a predicted condition "should only be performed when a therapeutic or prophylactic remedy is available, or when an estimate of the risk of transmission can assist parents in making reproductive decisions".

This is likely to be modified by pressure from individual WMA members. The British Medical Association, for example, pointed out in Bali that there are other advantages in predictive screening — such as suggesting possible changes in lifestyle in the face of a potential health threat — even for diseases that are not curable.

Others are expected to object to the harshness of a separate clause that warns that natural selection would be under threat if the various prophylactic measures made possible by knowledge of the human genome bring about the survival — and therefore the reproduction — of individuals presented altered genes.

Nevertheless, widespread support is likely among professional medical bodies for the general thrust of the statement, namely that doctors have a responsibility to ensure that care is taken when using the techniques of predictive medicine, to ensure individual patients are equipped to cope with the implications of the knowledge that these techniques can reveal. David Dickson

Call for partnership projects to join £80 million scheme

London. The UK government's bid to quell disquiet within the scientific community over its attempts to bring research closer to industry continued last week with the launch of an £80-million (US\$124-million) scheme to back research projects aimed at creating wealth and improving the quality of life.

The so-called Foresight Challenge, which was first announced in May and whose funding will be shared equally by government and the private sector, is being described as a competition to attract bids in line with the recommendations of the government's Technology Foresight exercise (see *Nature* 375, 265; 1995). The funding will last three years.

The competition was launched on Monday by Ian Taylor, the junior minister for science and technology. Earlier, at a press briefing, Robert May, the government's chief scientific adviser, invited "imaginative proposals" with an annual cost in the range of £1–2 million from groups comprising partnerships of industry and academia. Projects falling below this threshold will not be ruled out. But the upper limit of public sector contributions will be £4 million.

Short outlines of projects, he said, would be assessed in the first instance by a five-person panel including himself and the director general of research councils, Sir John Cadoogan. After the submission of fuller bids — in which the basic science in a project would be peer reviewed — the panel will present a provisional shortlist to the Foresight steering group, which will advise ministers on a final shortlist. Successful bidders will be notified in March next year. The call for a second round of projects will begin in June 1996.

Proposals did not have to be at the "near-market" stage, as this would imply that industry was already involved. "We are looking for groups of people who wouldn't nor-



May: seeking 'imaginative proposals'.

mally talk to each other, to stretch an idea to create a product," says May. "It's an incentive for researchers to look for industry, and for industry to look for researchers."

May adds that the government does not expect the exercise to result in "a product on the shelf" within three years. "But a fair fraction of them should have progressed from an idea to the point where you could begin ramping them up to a pilot project." He adds that there are no prescriptions for royalties and intellectual property rights. "It is up to the partners to hammer that out."

Signs are already emerging that Britain's research councils are shifting their funding priorities to tie in with the conclusions of the Technology Foresight, launched in 1993 in the wake of the government's white paper on science. Last week, for example, the Engineering and Physical Sciences Research Council (EPSRC) launched *Achievements in Chemistry*, a booklet highlighting the — distinctly applied — projects funded by the EPSRC in chemistry. An accompanying EPSRC statement pointed out that the research council's £33 million chemistry programme "is helping to support a thriving base for wealth creation". Ehsan Masood

Australian researchers prepare for strike

Sydney. Plans for an unprecedented half-day strike by scientists in a division of Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO) — believed to be the first strike against management in its 70-year history — point to continuing unrest in the country's main publicly funded research organization.

The strike is scheduled to occur this week, and involves scientists in the CSIRO's Division of Building Construction and Engineering in Melbourne. The stoppage has been called in response to a change in research priorities that will lead to the termination of various research programmes.

The division has 60 senior project officers, and of these about six or seven

scientists are to be redeployed as a result of the changes. Alan Reid, chief of the Institute of Minerals, Energy and Construction — which includes the building division — claims that the planned strike is "more of a protest rally". He says that both the staff and their labour union have been fully informed of the changes, which are being introduced as part of the division's efforts to respond to the needs of the market and Australian industry.

But Sandy Ross, a spokesman for the CSIRO section of the Public Sector Union, says that the staff are seeking clear written explanations setting out why proposals for the continuation of certain projects had been rejected.

Mark Lawson

French research budget wins increase, but few new posts

Paris. Research fared relatively well in France's austerity budget last week, with the government promising in particular to alleviate the funding crisis at the Centre National de la Recherche Scientifique (CNRS). But there is a virtual freeze on scientific recruitment.

Overall, the harsh cuts in public spending were not unexpected. France is struggling to reduce its spiralling budget deficit — which is estimated at more than FF322 billion (US\$64.4 billion) this year — in a bid to meet the criteria agreed under the Maastricht Treaty for participation in Europe's Economic and Monetary Union. The government is also under pressure to reduce both direct and indirect taxes, which at 44.7 per cent of gross national product are the highest of any industrialized country.

But Alain Juppé, the prime minister, refrained from making deep spending cuts, a decision that has been strongly criticized by the financial markets. The move was made partly to honour promises made by Jacques Chirac, the French president, during his election campaign earlier this year. Overall public spending is set to increase by 1.8 per cent next year, compared with a predicted level of inflation of 2.2 per cent.

Research emerges as one of the main winners in the budget, increasing by 2.4 per cent over the revised budget for 1995 — or 1.4 per cent compared with the previous government's request — to FF53.09 billion.

The increase is a personal success for Elisabeth Dufourcq, the new secretary of state for research. Many observers had questioned whether she would be able to defend research spending, often considered an easy target for the finance ministry in an austerity budget. "We can't say research has been badly treated", says Dufourcq.

Within the civil research and development budget (see figure below), the main research organizations receive large increases, confirming their role as the main forces

shaping French research. This confirms Dufourcq's confidence in the agencies, and her reluctance to increase the central role of her own ministry in shaping French research, a policy her predecessor, François Fillon, had seemed keen to promote.

The CNRS, for example, will have its funds increased by 3.66 per cent to FF13.1 billion, the national biomedical research agency, INSERM, by 5.27 per cent to FF2.25 billion, and the national agricultural research organization, INRA, by 5.97 per cent to FF3.3 billion.

Of the money allocated to CNRS, FF277 million is intended to alleviate the funding crisis at the agency. This time last year the agency told its researchers that they could spend no more than 60 per cent of the funds they had been allocated for the year (see *Nature* 371, 192; 1994), following the discovery of a gap in the agency's finances of more than FF1 billion.

This deficit had grown because cash payments from the government had failed to keep up with promises made in the budget. The new injection of funds follows an extra FF400 million provided earlier this year, and the agency now hopes to stabilize its financial situation in 1996.

Dufourcq also obtained an additional FF800 million for her ministry's own research funds, which had accumulated an even larger deficit than that of CNRS for the same reasons. The funds, which are used to support industrial and other special projects, had previously been "a target for the finance ministry", said Dufourcq.

Referring to the financial crisis at CNRS and her own ministry, Dufourcq says she inherited a financial situation that was "not brilliant". She also promises that the 1996 budget was free of the artificial promises that has brought about these crises. "I have decided to lance the abscess", she says.

But recruitment in the research sector became one victim of the emphasis that

Dufourcq placed on rectifying the financial situation.

As a result of the financial stringency, only eight new posts will be created next year for the whole of the scientific community.

"It wasn't the priority," said Dufourcq.

Declan Butler

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Lapping it up: a Hindu statue 'feeds' on milk.

India's 'milk miracle' is hard to swallow

New Delhi. Science took a hammering from religion last week when millions of Hindus packed temples all over the world to offer milk to Ganesha, the elephant god. They had heard — by word of mouth, over the radio or by telephone — that the stone idols were drinking milk by the spoonful, a manifestation of the presence of God himself.

Many of those who lined up to spoon-feed the deities watched the milk disappear as soon as it came into contact with the wet surface of the idols' trunks. The unlucky ones whose spoons remained filled had to swallow the priestly admonition that their belief was not strong enough.

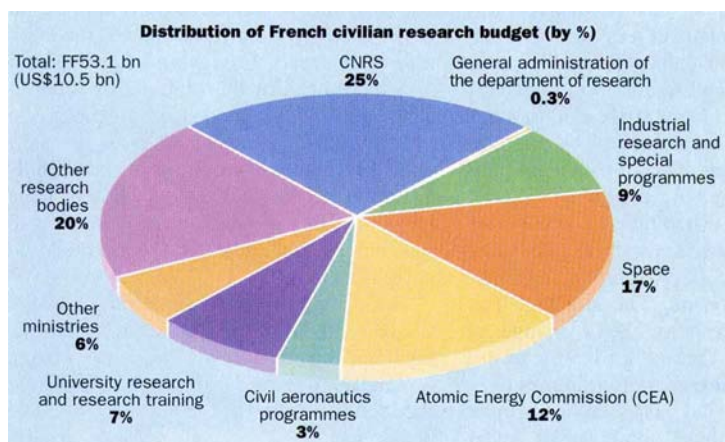
But the All India Scientists and Rationalists Association, based in Calcutta, dismissed the 'milk miracle' as a hoax. "What people saw was simply a demonstration of capillary and surface tension at work," explained Professor Yash Pal, the government's former science secretary.

Scientists at the National Council for Science and Technology Communication added a red dye to milk and showed the coloured milk being sucked in by capillary action dripping from an idol's body. Leading scientists in Madras signed a statement calling on educated Indians to ensure "that primitive obscurantism and superstition did not hold sway over a society on the threshold of the 21st century".

The Delhi Science Forum, an organization committed to improving scientific literacy, described the apparent miracle as a well-planned conspiracy designed to arouse communal emotions. The forum suggested that people with a knowledge of physics may have introduced spoon-feeding "to create the conditions for the miracle to occur".

Few know how the affair started. But the ruling Congress government blamed the opposition pro-Hindu Bharatiya Janata Party for spreading the rumour in order to use the religious feelings aroused to gain support in the forthcoming elections.

The Vishwa Hindu Parishad, self-styled Hindu guardians, claimed the 'miracle' signalled a Hindu revival. **K. S. Jayaraman**



Euro-MPs turn up the heat on energy projects

Munich. The European Parliament's committee on research, technological development and energy has begun an investigation into allegations that the European Commission has mishandled money intended for research into renewable energy sources.

The committee plans also to use the opportunity to raise with the commission the wider question of the lack of openness in the commission's procedures for allocating research money, which some claim leads to a climate of distrust.

The allegations focus on the Joule programme, which has been allocated ECU967 million (US\$1.3 billion) to distribute over five years. The commission has agreed to devote about 60 per cent of the programme's funds to projects concerned with renewable energy resources, such as solar and wind power. But only 40 per cent of the ECU191 million distributed in the first round in July went to renewable energy projects (see *Nature* 376, 628; 1995).

In a statement issued last week, the commission rejects claims that the views of outside experts were manipulated during the review process. Jean-Christophe Filori, a spokesman for the commission, says that there were not enough good renewable energy projects this time round. But he adds that the balance between renewable and conventional projects will be corrected during future funding rounds.

Filori claims that the commission has

"absolutely no political wish to downgrade renewable energy in Europe". But members of the European Parliament (MEPs) are not satisfied with this reassurance. They argue — and the commission acknowledges — that some of the renewable energy projects were downgraded from an initial rating after they had been assessed by two independent

IMAGE
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Renewable energy: solar cells at Adrano, Sicily.

expert committees, one looking at the scientific merits and the other at the strategic merits of applications.

Nuala Ahern, a Green MEP for Ireland, says the commission has not yet provided a satisfactory explanation of why the grades had been changed. "The European commission sets the agenda and then moves the goal posts", she says.

The British, Greek and Irish members of the committee overseeing the Joule programme, which is made up of representatives

from each European Union state and is invited to give its opinion on the final list of projects put forward by the commission, have also complained.

David Irving, for example, who is head of the Department of Trade and Industry's environment and energy technologies division, and the UK representative on the committee, said in a letter to the commission that the United Kingdom was unable to support the commission's proposed list, "not because of misgivings over the 181 projects *per se*, but over the manner by which the list had been derived".

In particular, wrote Irving, the British delegation felt that "some kind of re-examination of the 'downgraded' projects was needed to reassure the research community about the fairness of procedures".

One of the three members of the Joule expert committee concerned with assessing the strategic aims of applications was Jört Schindler, manager of the Munich-based company Ludwig Bolkow Systemtechnik GmbH. He says the experts were very unhappy that the gradings of applications were changed without consultation.

Filori denies that the programme has been manipulated. "In the fourth Framework programme there are a lot of very strict criteria to be applied to grant applications, in addition to scientific excellence, which are not widely understood", he says.

Alison Abbott

Astronomers view 'tolerance' satellite with jaundiced eye

Boston. Plans to launch a bright satellite to celebrate the proclamation by the United Nations of 1995 as the "Year of Tolerance" have brought protests from both the American Astronomical Society (AAS) and the International Astronomical Union (IAU).

According to current designs, the proposed satellite, known as the 'Star of Tolerance', would consist of two giant, reflective balloons, of 30-metre and 50-metre diameters respectively, linked by a 2-km tether. The structure would circle the planet every two hours, and would remain in low Earth orbit for between 18 months and four years before burning up in the atmosphere.

Visible to the naked eye in the hours before and after sunset, the satellite is intended to remind those who see it to accept cultural difference both between and within communities. The venture is led by Nersi Razavi, a Paris businessman, and has already won the support of the United Nations Educational, Scientific and Cultural Organization (UNESCO).

A launch date has not yet been fixed, although the Russian space agency has offered to carry the balloon payload at a

total mission cost of about \$20 million. "We're shooting for the end of 1996, but that depends on many factors, including money," says Serguei Lazarev, coordinator of the project for UNESCO. Various fund-raising options are being considered, including the idea of a 'world lottery'.

But astronomers hope to intervene before the project goes much further. "We applaud the worldwide recognition of world peace and tolerance of all peoples, but the proposed manner of communicating these ideas would seriously impair the science of optical astronomy all over the world," wrote Robert Milkey, executive officer of the AAS, on 29 August in a letter to Federico Mayor, UNESCO's director general.

"This is not the first time an idea like this has been raised," says Woody Sullivan, an astronomer at the University of Washington who is vice president of an IAU commission on protecting observatories from optical and radio interference and space debris.

In the late-1980s, for example, the French government backed a plan to orbit a 24-km-circumference band of reflective balloons, a so-called 'Ring of Light', to commemorate

the 100th anniversary of the Eiffel Tower.

None of these plans has yet materialized, and Sullivan would like to keep it that way.

Patrick Crane, chairman of the AAS committee on light pollution, agrees. "Much pain could be avoided if the sponsors of these projects were to consult with astronomers ahead of time, so that we didn't go over the same lessons each and every time." The basic problem, he says, is that unlike the use of the radio spectrum, which is governed by the International Telecommunications Union, there are no regulations on space art or space advertising and no formal agencies equipped to deal with these questions.

UNESCO's Lazarev maintains that he has seen several letters from astronomers claiming that the level of brightness from the Star of Tolerance would not disturb scientific observations, although he accepts that this position is far from unanimous. "The most important thing about this mission is the symbolic message," he says. "Otherwise, the project is entirely flexible. The design of the satellite can be changed, and its brightness adjusted, to make sure it doesn't contribute to space pollution."

Steve Nadis

Whaling dispute continues

SIR — Apparently alarmed that an advertisement in *Nature* referencing the study of Baker and Palumbi (*Science* 265, 1538; 1994) might lend undeserved credence to the notion that illegal whale products find their way into Japanese markets, Milton Freeman (*Nature* 376, 11; 1995) reiterates the arguments of the Fisheries Agency of Japan (FAJ) that the study is fundamentally flawed. As Freeman's letter contains several serious errors, we feel obliged to comment.

First, Freeman states that "the meat of all species referred to by Baker and Palumbi could be legally imported into Japan until 1991". That is not true. The humpback whale was fully protected worldwide in 1966. To be legal, the humpback meat would have to have been stored for 30 years. At the meeting, one of us (R.L.B.) suggested an alternative explanation; the humpback meat came from a Japanese stranding or an incidental fishery kill that was processed because of the high value of whale meat.

Second, Freeman says that Baker and Palumbi were "seriously questioned" at a recent international symposium on marine mammal genetics; Freeman himself was not there. Two of us (A.E.D. & W.F.P.) convened that symposium and Freeman is correct. The paper was seriously questioned — but only by N. Yagi of the FAJ. But the balance of the meeting participants accepted the methodology and results.

Third, Freeman misunderstands genetic methodology. Baker and Palumbi did not claim to find, as Freeman states, "an mtDNA sequence midway between a minke and a humpback".

Rather, two sequences were derived from amplification of two pieces of marinated meat from a single package; nothing was "midway". The authors' suggestion, that the product contained meat from two different sources, is entirely plausible. Freeman goes on to attribute, using quotation marks, the words "intermediate between a sperm whale and a harbour porpoise" to Baker and Palumbi, although this phrase does not appear in their paper. We trust this was a typographical error by Freeman and not an attempt to bolster his poor argument by incorrectly attributing a faintly absurd statement to the authors.

Our laboratory possesses a type sequence catalogue of all but 22 of the 80 or so extant cetacean species and we have extensive experience with intraspecific sequence variation in large datasets. Given a sequence of reasonable length, and ignoring rare interspecific hybridization in the wild of closely related species, we find (as do others) that identification to species of an unknown sample is a high-

ly practicable undertaking. With or without a complete collection of type specimens, mitochondrial DNA control region sequences easily discriminate between baleen and toothed whale samples. Sequences from different species do not "converge" upon one another. Thus Freeman's implication that the lack of type sequences for the toothed whales sold in Japanese markets could result in the incorrect identification of some baleen samples is entirely without foundation.

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ORI's unhappy lot

SIR — John Maddox's account (*Nature* 376, 721; 1995) of the ORI/AAAS Conference on Plagiarism and Theft of Ideas was based on the ORI (Office of Research Integrity) report of the conference and misses the emotions at the event. Policemen were in the auditorium. At the time of the meeting, ORI was still concealing the fact that it would not prosecute supervisors for stealing ideas from their underlings. Jane Rosen made pointed comments from the floor about how her university and ORI had dealt with her case against her PhD supervisor.

Many in the audience were irritated to hear speakers talk of rules and procedures when it was obvious that cases were most often decided by political clout within the science community. I said from the floor that ORI's freedom of action was limited, and that the last two directors of its predecessor OSI lost their jobs after doing two vigorous investigations (of the Baltimore affair and the Gallo case). Lawrence Rhodes of ORI, on the podium, said I had insulted him. My remark and his response do not appear in the report.

The audience understood that the lot of ORI people is not a happy one. When I later said we should all thank them for being willing to "expose themselves to this angry mob" there was a roar of applause. My remark was altered in the report. "Angry mob" was omitted, leaving no reason for the applause, which was still mentioned. It may now be impossible to learn how much was changed between audiotape and report because ORI says it reused the tape and discarded the computer cassette transcribed from it.

Charles W. McCutchen

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No budget cuts

SIR — I am surprised that you should have published (*Nature* 376, 718; 1995) a spurious story about alleged budget cuts based on a leaked letter from me. On 16 February 1995 I wrote to the research council chief executives advising them of figures to be used in future planning exercises. These figures were subsequently announced to parliament on 27 February in answer to a parliamentary question (Hansard, House of Commons Reports, Vol. 255, col 425, 27 February 1995).

These figures have therefore been in the public domain for more than six months and have been discussed already in the appropriate journals. There has been no secret about this, nor is it secret that it is the government's intention that all publicly funded research programmes should take account of the findings of the Technology Foresight exercise. The allocations of the Science Budget announced on 2 February for 1995–96 included some special initiatives designed to strengthen the science base in particular areas; the planning figures merely carried this forward. There is no justification for referring to these initiatives within the research council system as cuts to the science budget.

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Factual errors

SIR — In his review of my book *The Price of Greatness: Resolving the Creativity and Madness Controversy* (*Nature* 375, 547; 1995), Stuart Sutherland comments that my regression analysis correctly placed 95 per cent of my subjects and then says that 75 per cent could have been correctly placed on the basis of gender alone because "three times as many men as women were in the top quartile". This statement contains three factual inaccuracies. First, the analysis correctly placed 92 per cent of subjects, not 95 per cent. Second, three times the percentages of men as women — not three times as many — were in the top quartile. Third, gender alone does not accurately predict 75 per cent of the cases; it predicts only 63 per cent, about the same percentage as mental disturbances alone. Such careless errors are unexpected in a scholarly book review.

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■ We regret the factual errors. — Editor, *Nature*.

A big book of the human genome

Nature's Genome Directory, the largest compilation yet of published data about the human genome, appears today. Here Peter Goodfellow discusses the first paper, which deals with the information to be gleaned from almost 300,000 partial sequences of complementary DNA (expressed sequence tags or ESTs). Overleaf, Peter Little assesses the mapping studies presented in the other five papers in the directory.

Complementary endeavours

Peter Goodfellow

"THE development of methods of cloning and sequencing DNA has liberated genetics from the constraints of breeding experiments, and all organisms are now accessible to genetical analysis by the new methods"¹. Ever since Sydney Brenner first argued for a programme to sequence random complementary DNA clones to identify new genes, this approach has been surrounded by dissension, and the name expressed sequence tags — better known by its acronym ESTs — is almost synonymous with controversy². The publication from Adams *et al.*³, in the *Genome Directory*, of 52 million base pairs of EST sequences will allow a rational evaluation of this type of data.

Only 3 per cent of the human genome codes for proteins; what the rest does is unclear, but much of it may have no function. The new EST philosophy follows Brenner's argument that the most important part of the genome is the protein-coding genes and that sequencing these will give the largest return of biological information per base pair of sequence.

Few biologists will disagree strongly at this point. Rather, the disagreement starts with the extrapolation of the argument to random partial sequencing of genes. The easiest way to access protein-coding sequences is to sequence cDNAs. The EST philosophy extends the cost-effective argument one step further by suggesting that the maximum return on sequencing comes from a single partial sequence or 'tag sequence' for each gene, hence the name 'expressed sequence tag'.

Intuitively, this feels wrong and many people, myself included, sniggered over this apparently simplistic philosophy. An average cDNA is a few kilobases in length; an average 'tag' produced using a sequencing machine is 200–300 base pairs. In effect only a small part of the coding sequence is identified for each gene, the same gene may be 'tagged' more than once without the investigator realizing and the single sequencing run will have sequencing ambiguities. Furthermore, cDNA libraries reflect the messenger RNA they are prepared from; abundant transcripts will be sequenced multiple times, producing no new information, and

rare transcripts will be missed. Given these disincentives, why have tens of millions of dollars been spent on ESTs?

After a brief exploratory phase, in which a few thousand ESTs were produced^{4,5}, a major driving force was the hope and the fear that ESTs could be used to patent any gene that had been tagged. Attempts to patent genes via ESTs raised the spectre of an international trade war and the echoes are still reverberating around courts in the United States. Discounting the politics and the stock market, the predicted scientific benefits were of two types. It was hoped that large-scale EST projects would define the broad limits on the number of genes and the types of genes in the genome and provide a snapshot of their expression patterns. It was also expected that enough sequence information would be generated to be able to recognize new members of gene families of biological and commercial interest.

The paper by Adams *et al.* fulfils many of these predictions. By estimating the fraction of newly generated ESTs which match already described genes, it is possible to make an estimate of the total number of genes in the human genome. The

number predicted is consistent with the generally held prejudice of around 100,000. The EST information can also be used to estimate the number of gene families and this in turn can provide a guide to the number of genes involved in different roles in the cell. I am not certain that this information is either robust or very useful as it makes all sorts of assumptions about our current knowledge of cell biology; nevertheless, these data will probably appear as dogma in high-school and undergraduate textbooks.

I also have reservations about the conclusions based on the number of times an EST appears in a cDNA library. This information is obviously useful for genes expressed at high levels but if the number of ESTs produced from each library is of the order of a few thousand, the sample error for genes expressed at levels below 0.1 per cent is enormous. The large majority of genes are expressed at these low levels and deductions about where genes are and are not expressed must be drawn cautiously.

Nevertheless, I have to confess that I am now convinced of the usefulness of EST data. The sequence generated is an important source of information about genes. Electronically screening large EST datasets with fragments of sequence can identify new cDNAs for a gene of interest and can help to identify closely related genes. This utility explains the success of

Availability

The *Genome Directory* is a supplement to *Nature*, published separately, and is being mailed direct to all institutional subscribers along with this issue. Personal subscribers are not being sent the directory in this way, in the belief that many will have no need of it. They can, however, obtain it at no cost by writing to: Marketing Department, *Nature*, Porters South, Crinan Street, London N1 9XW, UK, quoting their subscription number.

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the public-domain database dbEST⁶, which contains some of the data produced by Adams *et al.* and over 170,000 ESTs produced at Washington University by a consortium funded by Merck. Since its inauguration, dbEST has been screened

over 200,000 times and the rate of screening is increasing exponentially (M. Boguski, personal communication).

There are two ways of increasing the value of ESTs. The first is to map the position of the corresponding gene in the genome¹. This can be done either by assigning ESTs to already mapped contigs⁷ such as those described in the *Genome Directory*; another method is to use whole-genome radiation hybrids⁸. It is likely that both methods will be used in parallel to produce a 'transcript map' of the genome^{9,10}, which will greatly simplify positional cloning of genes of biological and medical interest. The second way to add value is to use the ESTs as a starting

point to produce complete sequences of gene transcripts. Adams *et al.* are attempting to do this by screening ESTs against each other and using overlapping sequences to produce full-length transcripts. It is too soon to conclude whether this method is more cost effective than direct sequencing.

The production of a public-domain transcript map, annotated with complete gene sequences, is a task worthy of the complementary endeavours of all who map and sequence genes. □

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Navigational progress

Peter Little

"ANOTHER damned, thick, square book! Always scribble, scribble, scribble! Eh! Mr Gibbon?" The Duke of Gloucester's words to Edward Gibbon in the eighteenth century might well apply to the latest batch of results from the Human Genome Project which are presented in the *Genome Directory*.

This is the largest body of information about the physical structure of our genetic apparatus that has ever been published: it is a snapshot of the genome project at a critical juncture. It is also probably the last time that paper will be an adequate medium to publish results: the Duke was more prescient than his boorish comment might have suggested.

The bulk of the directory presents physical maps of the human genome: the key technology used to construct these is yeast artificial chromosome (YAC) vectors to separate the DNA of the human genome into fragments of 200,000–2,000,000 base pairs. These fragments are then laboriously analysed to work out which fragment is adjacent to which. The ultimate goal is to have a complete representation of the genome in overlapping sets, called contigs, of YAC clones, reducing the 3×10^9 base pairs of the genome to smaller, more manageable, units.

The largest body of work in the directory comes from Centre d'Etude du Polymorphisme Humain in Paris and collaborating groups (Chumakov *et al.*¹) who are using factory-scale facilities to analyse the whole genome. They have identified 225 contigs, with an average size of 10 million base pairs and covering perhaps 75 per cent of the genome. Contigs were identified and constructed using a mixture of techniques: sequence tagged site (STS) content mapping (see figure), based on the polymerase chain reaction, and fingerprint and hybridization analyses. The raw data required sophisticated

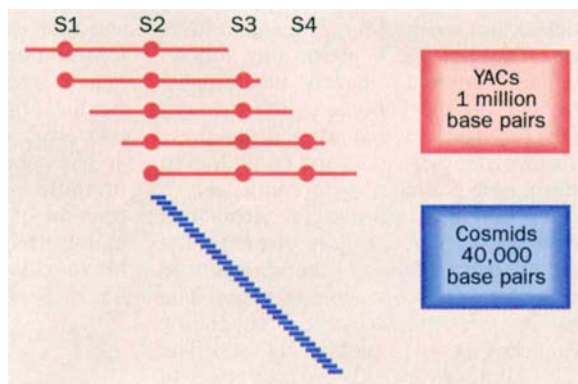
computer analyses to be assembled into contigs.

Remarkably, this analysis really is not sufficient, as evidenced by the papers from Gemmill *et al.*², Krauter *et al.*³ and Collins *et al.*⁴. These report even more detailed analyses of chromosomes 3, 12 and 22, incorporating very large sets of data from outside of the analysis of Chumakov *et al.* These 'second-generation' maps, constructed by simply using a higher density of STS and hybridization markers, are at a greater resolution than could easily be achieved by the whole-genome approach. The work of all these groups could not be achieved without a solid framework of ordered markers which has been generated not only on a genome-wide basis⁵ but also by the many 'single-chromosome' labs working in international consortia. The second-generation maps are further proof that free collaboration, not competition and secrecy, is the only way that real progress can be made within the Human Genome Project.

The other paper in the physical mapping area of the *Genome Directory* comes from Doggett *et al.*⁶ and reports a physical map of chromosome 16. This paper is very important not just as a map of chromosome 16, but because it starts to describe the assembly of maps at even higher resolution: 40,000 base-pair fragments cloned in cosmid vectors. These clones are the substrates for the next phase of the Human Genome Project — the establishment of the DNA sequence, and the

complexities of assembling contigs at this resolution are far from trivial.

Maps for other chromosomes are being made: those for chromosomes 19, 11 and 7 are all far advanced, and perhaps half the genome is covered by single-chromosome interest groups. How these groups will interact with the whole-genome analyses and with the large-scale sequencing operations will depend, in part, on how money is allocated by the funding agencies. What emerges with real clarity in these papers is that to divorce the process of map assembly from the communities that work intensively on the genome —



The chromosome maps consist of yeast artificial chromosomes (YACs, red), the relative position of which is established by the presence or absence of a sequence tagged site (S1 to S4) within their DNA. Establishing a map of cosmids (blue) will require the analysis of 25 times more cosmids than the number of YACs in the map.

the single-chromosome groups — is impossible. The knowledge that goes into error checking and trapping requires a detailed understanding of chromosome maps: applying this knowledge was how the second-generation maps were developed, and the dilemma for the funding organizations is how to reconcile the need for the economies of scale with the disparate location of the pertinent information in the single-chromosome groups.

What remains to be done? A massive task must be the construction of cosmid-level maps of the genome (see figure);

sequencing will follow closely in the footsteps of the mapping analysis and will also contribute directly to this programme. A more immediate decision is to what extent genes should be placed on the physical map, to make the maps biologically informative, before sequencing starts. The other paper in the *Genome Directory*, that by Adams *et al.*⁷ which is discussed by Peter Goodfellow on page 285, dramatically highlights this point. In part, this process is already underway — many cDNAs and coding sequences have been positioned by hybridization or STS content mapping, but the number of genes that remain to be placed is enormous.

It would be a great mistake to see the approach of enumeration of coding sequences as a substitute for the goal of the Human Genome Project. This goal is explicit — to understand the information content of our DNA. To do this, we need to know the sequence not just of coding regions: we need the sequence of the DNA that controls the flow of information: of promoters, controlling elements, enhancers, locus control regions and so on, all of which are missing from cDNA. Complementary DNA is not the same as genes, and it is genes that we need to know about. We have little or no information about where controlling regions lie within vertebrate DNAs, so even if we had the technical ability to know where genes are — which we do not — we still would not know where to sequence in relation to the gene.

What we see in the mapping papers published in the *Genome Directory* is the necessary first step to understanding the information content of the genome. The publication⁸ of the first sequence of the genome of a free-living organism, that of *Haemophilus influenzae*, and the rich source of biological deductions that can be made from this sequence, will be replicated a thousand-fold in the vastly more complex human genome. The fact that we cannot yet understand the complexity of DNA does not mean we shouldn't assemble the information. And the amount of information is huge, as the *Genome Directory* graphically illustrates. Which is where another damned, thick, square, book — or more probably its electronic equivalent — will come in. □

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Bullets or fragmenting shells?

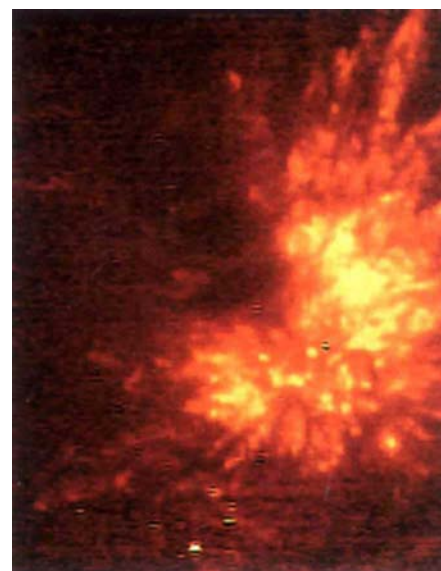
Mordecai-Mark Mac Low

Two years ago, this journal published spectacular images by Allen and Burton¹ of a wide fan of linear features in the Orion nebula, apparently produced by high-velocity 'bullets' of interstellar gas explosively ejected from a massive young star named IRC2. On page 315 of this issue, Stone *et al.*² propose instead that a strong stellar wind from IRC2 sweeps up a smooth shell of interstellar gas, which is then fragmented by gas dynamical instabilities as the stellar wind velocity increases on a timescale of a few hundred years. Recent observations give startling confirmation of this proposal.

The bullet explanation has been proposed repeatedly over the years as an explanation for observations of bow shocks, similar to the sonic booms from supersonic planes, everywhere from star-forming regions to supernova remnants. In every case, the question that remained open was how small clouds of dense gas could be accelerated up to high velocities without being disrupted. Stone *et al.* draw together theoretical work — mostly computational — over the past decade to show that such bullet-like objects in astrophysical flows are almost always generated by gas dynamical instabilities in initially smooth winds and jets. Gas is first accelerated, by more or less well understood mechanisms, and only then formed into discrete clumps that drive observable bow shocks into the interstellar gas as they decelerate.

The central idea in the proposal made by Stone and co-workers is that massive young stars might have strong stellar winds with velocities that vary over time. Although it is a plausible idea, little direct evidence for quasi-spherical stellar winds from extremely young stars yet exists. In their picture, an initial slow wind drives a shock into the surrounding interstellar gas, sweeping it up into a thin, dense shell, and forming a classic stellar wind bubble³, which decelerates as it continues to sweep up more gas. As long as the shell continues to decelerate, the dense shell effectively supports the rarefied interior, so the interface between them is stable to Rayleigh-Taylor instabilities — the overturn seen when, for example, dense vinegar is supported by less dense oil. At some point, the star begins to blow a faster wind, suddenly accelerating the shell, so the rarefied interior of the bubble now supports the shell. Strong Rayleigh-Taylor instabilities then set in, fragmenting the shell. Stone *et al.* show with their numerical computations that the fragments then indeed behave like bullets, trailing long bow shocks in their wakes.

The Orion nebula lies on the front surface of a dense molecular cloud, known as OMC-1, that contains the nearest region currently forming massive O and B stars. The most massive of these appears to be IRC2, with a luminosity as much as 10^5 times that of the Sun⁴. Because of the obscuration of dust in the cloud, the star-forming region can only be observed at infrared and longer wavelengths. Over 15 years ago, observations in emission lines such as the 2.122- μm transition of H_2 showed that a thin layer of shocked H_2



Infrared image of Orion, showing emission from shocked H_2 . Bullets can most clearly be seen in the upper right and lower left, while the striated emission in the centre appears remarkably similar to that predicted by Stone and co-workers². (Courtesy of M. J. McCaughrean, Max Planck Institute for Astronomy.)

covered much of the region⁵ around IRC2. Higher-resolution observations in this line revealed the bow shocks and 'bullets' discussed here^{1,6,7}.

The figure shows a new image in the line, shown here in false colour and logarithmically scaled to enhance the fainter features. The data were obtained by Mark McCaughrean (personal communication) using the MAGIC camera/spectrometer on the Calar Alto 2.2-m telescope. A series of long-slit spectra were taken stepping across the IRC2 region one slit at a time. The H_2 line data were then extracted, allowing this accurately continuum-subtracted image to be reconstructed. Similar results have been obtained using a Fabry-Perot imaging spectrometer by Schild *et al.*⁸. The earlier observations^{1,6,7} of the outer finger-like structures on which Stone *et al.* based their work did not

1. Chumakov, I. M. *et al. Nature* **377** Suppl., 175–297 (1995).

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5. Gyapay, G. *et al. Nature Genet.* **7**, 246–339 (1995).

6. Doggett, N. A. *et al. Nature* **377** Suppl., 335–365 (1995).

7. Adams, M. D. *et al. Nature* **377** Suppl., 3–174 (1995).

8. Fleischmann, R. D. *et al. Science* **269**, 496–512 (1995).

show the fragmented nature of the bright central region. However, the highly fragmented shell seen here is very naturally predicted by the model of Stone *et al.*, giving strong support to their proposal.

Some sort of large stellar wind bubble has long been thought to be producing the emission from shocked H₂ in Orion. These new observations and computations appear to make much clearer the nature and extent of the bubble, and perhaps tell us something new about massive young stars — namely that they have strong and varying stellar winds, in addition to the well known collimated jets. It will be interesting to see how well models of young stars can accommodate the need to produce these winds.

Although Orion provides an exciting and nearby example, Stone *et al.* point out that Rayleigh–Taylor and other dynamical instabilities drive similar processes of bullet formation in many other situations. Perhaps the object with the most striking resemblance to OMC-1 is the Homunculus nebula around η Carinae — a massive post-main-sequence star — which can be modelled along very similar lines⁹. Herbig–Haro objects produced by protostellar jets are another example of isolated, bullet-like objects produced by the fragmentation of a shell swept up by a stellar outflow¹⁰. Rayleigh–Taylor instabilities occurring during the actual explosions of supernovae appear to produce dense clumps of material that may thereafter indeed act as interstellar bullets¹¹, as observed in supernova remnants such as Cas A and Vela. (Stone *et al.* offer an alternative explanation for the Vela bullets involving dynamical instabilities in a pulsar-driven bubble.) On even larger scales, starburst galaxies such as M82 seem to be driving clumpy shells into the intergalactic medium, because of Rayleigh–Taylor instabilities occurring as the shells accelerate out of the disks of the galaxies. Using the same physical explanation for objects over so broad a range of scales is an elegant feat. □

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Notch takes a short cut

Stephen Goodbourn

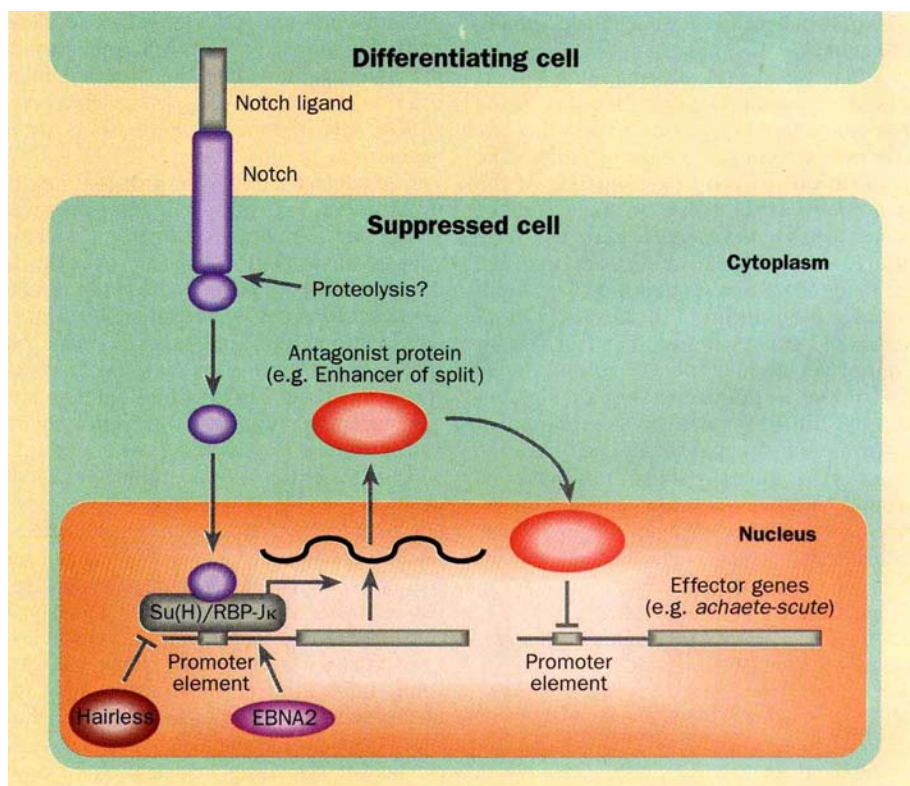
A MAJOR goal in cellular and molecular biology is to link events at the cell surface with specific changes in the pattern of gene regulation. In most cases signalling seems to be achieved using cascades of protein kinases, but Jarriault *et al.* (page 355 of this issue¹) now provide evidence that events initiated by occupation of the Notch receptor may dispense with a classical signal-transduction pathway altogether.

Notch and related proteins are found in both invertebrates and vertebrates, and are of especial interest to developmental biologists. In *Drosophila melanogaster*, the *Notch* gene plays a key role in lateral specification (reviewed in ref. 2). This is the process whereby a single cell differentiates from amongst a background of equipotent neighbours, as typified by the generation of neuroblasts from the ventral ectoderm of the *D. melanogaster* embryo. It is believed that a stochastic process leads a single cell to express a membrane-bound ligand for Notch. The *Notch* gene itself encodes a membrane-bound receptor that becomes activated upon ligand binding and signals the suppression of differentiation.

Consistent with this, null mutations in *Notch* result in all cells in the ectodermal monolayer developing into neuronal-like tissue. Neuroblastic differentiation is associated with a requirement for pro-neural genes such as the products of the *achaete-scute* gene complex which encode tissue-specific transcription factors. The *achaete-scute* proteins are expressed in all cells in the ventral ectoderm, but during normal development expression becomes restricted to the neuroblasts. Activation of Notch suppresses the expression of *achaete-scute* genes by inducing the synthesis of a set of transcriptional repressors encoded by the *Enhancer of split* (*E(spl)*) genes.

Notch is also involved in other cell-fate decisions in *D. melanogaster*, and *Notch* homologues may well have similar functions in other organisms. Notably, mammalian forms of activated Notch antagonize MyoD-induced muscle differentiation^{3,4}. The myogenic properties of MyoD are also antagonized by a mammalian homologue of the *E(spl)* genes called *HES-1* (ref. 5).

The induction of *E(spl)* transcription in response to Notch activation requires the



Model for induction of transcription by activated Notch. Upon ligand binding, the intracellular domain of Notch is released as a result of proteolytic processing and migrates to the nucleus where it binds to the product of the *Suppressor of Hairless* gene (*Su(H)*) or its mammalian equivalent *RBP-Jκ*; the upshot is transcriptional activation of genes that encode antagonists of differentiation. *Su(H)* is also negatively regulated by the product of the *Hairless* gene, and *RBP-Jκ* is positively regulated by the Epstein–Barr virus protein *EBNA2*.

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product of the *Suppressor of Hairless* (*Su(H)*) gene. The *Su(H)* protein is closely related to a mammalian DNA-binding protein called RBP-J κ , which is necessary for immunoglobulin gene rearrangements⁶ and expression of MHC class I genes⁷, and to mediate the transactivation properties of the Epstein-Barr virus protein EBNA2 (refs 8–10). The DNA-binding specificity of RBP-J κ has been established¹¹, and *E(spl)* and *HES-1* genes contain target sequences for RBP-J κ and, by analogy, presumably *Su(H)*, suggesting that *Su(H)* is the transcription-factor endpoint in the signal transduction pathway in response to Notch activation.

In an attempt to understand the Notch signalling pathway in mice, Jarriault *et al.*¹ studied the regulation of the *HES-1* gene. The authors show that expression of RBP-J κ either alone or in the presence of full-length Notch cannot stimulate *HES-1* transcription. However, it has been previously shown that removal of the transmembrane domain of Notch leads to constitutive activation in *D. melanogaster* (reviewed in ref. 2). Equivalent truncations of mammalian forms of Notch can inhibit myogenesis^{3,4}, and are associated with several neoplasias^{12,13}. These truncated forms localize to the nucleus, and in the case of myogenesis that event is essential for function⁵.

When Jarriault *et al.* tested truncated forms of Notch they found that they strongly stimulated the *HES-1* promoter, in a manner that depended on the binding of RBP-J κ . Activated Notch cannot directly bind to DNA, but forms a tertiary complex with RBP-J κ and DNA through a stable interaction between the two proteins. So it seems the RBP-J κ functions as a docking protein that directs the activated Notch to promoter targets, eliminating the need for intermediate factors (see figure). One possible mechanism for the activation of Notch invokes a conformational change induced by ligand binding that exposes the intracellular domain to a protease. What remains to be provided is unambiguous evidence that membrane-bound Notch is pro-

cessed in response to ligand binding.

The simplicity of this signal transduction pathway suggests that it may be very ancient. This is supported by the pleiotropic nature of Notch function, and the conservation of the pathway components from flies to mammals. Although there are many examples of ligands that can freely diffuse into cells and directly activate transcription factors (steroids and their receptors for example), there are also some precedents for signal transduction pathways that lack intermediates between the membrane and target genes. Sterol regulatory element-binding protein 1 exists in membrane-bound form (albeit bound to the nuclear membrane and the endoplasmic reticulum) and yet can respond to depletion in sterols by undergoing proteolytic cleavage to yield a soluble product that can enter the nucleus and stimulate transcription from specific pro-

motors¹⁴. Even more dramatically, lactoferrin released from secretory granules of neutrophils in response to infectious pathogens can cross the plasma membrane and migrate to the nucleus of nearby cells, thereby activating specific transcription¹⁵.

It remains to be seen how common such simple pathways are. Although they suffer the disadvantage relative to the common protein kinase cascades of not being amplificative, they offer the advantage that they are not subject to cross-talk and interference. They might therefore offer the exquisite specificity that may be vital in some circumstances, particularly in early development. □

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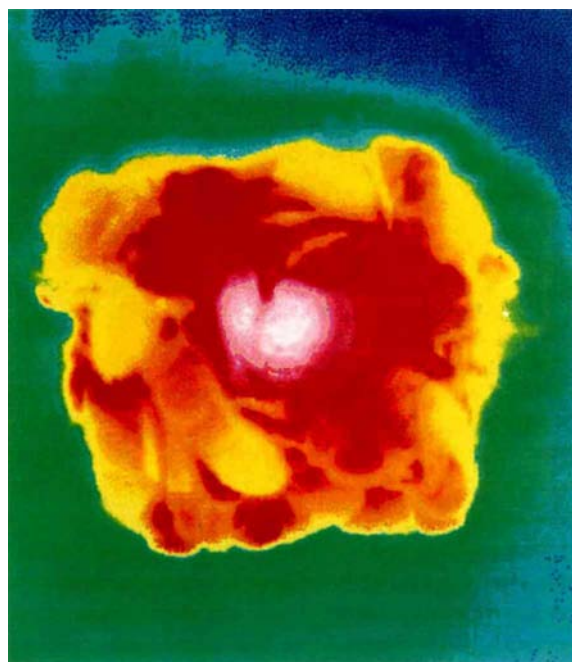
ANIMAL BEHAVIOUR

Neighbourhood watch

THIS picture illustrates one of the more spectacular weapons used in the unceasing war between Japanese giant hornets (*Vespa mandarinia japonica*) and honeybees (*Apis cerana japonica*), as graphically described by Masato Ono and colleagues of Tamagawa University on page 334 of this issue. It is a thermogram of a hornet being engulfed and baked to death by a ball of angry bees.

What has the hornet done to incur such displeasure? Japanese hornets are insatiable predators of smaller bees and wasps, raiding nests for larvae and pupae which they carry off to feed their own young. Ono and colleagues report that a raiding party of as few as 20 hornets can kill as many as 30,000 bees in three hours, laying waste to entire hives. The hornets can then occupy the hive at their leisure, picking off the defenceless larvae. Introduced European honeybees (*Apis mellifera*) are easy prey to this take-no-prisoners strategy, but native bees have evolved suitably drastic countermeasures.

Bees are alerted when hornets on reconnaissance missions stop at hives to mark them with a distinctive pheromone. Unlike European bees, Japanese bees have long been able to decrypt



messages in hornets-only code, and lie in wait for the inevitable raid. Individual bees are no match for a hornet in single combat — a coordinated defence is required, and bees are masters at this kind of organization. On sighting a hornet, bee look-outs signal to their colleagues, who lie in wait just inside the entrance to the nest. Should the hornet try and enter, it rapidly disappears within a ball of some 500 bees. The temperature within the ball — as shown in the accompanying picture — rises to about 47 °C, which kills the hornet but leaves the more heat-tolerant bees unscathed.

Henry Gee

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Blindsight in real sight

Alan Cowey

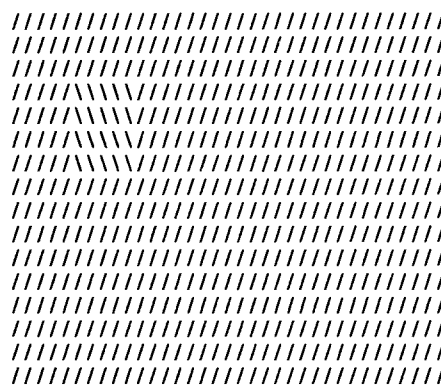
SOME brain-damaged patients with defects in their visual field manifest the extraordinary phenomenon of blindsight — they can detect, localize and even identify visual stimuli that they deny seeing. Their considerable residual visual processing must be demonstrated instead by forced-choice guessing, which the patient often dislikes and believes to be pointless, perhaps explaining why blindsight was not discovered much sooner¹. On page 336 of this issue² Kolb and Braun describe an experiment, using forced-choice methods, that demonstrates blindsight in normal vision and provides clues as to its nature in the absence of the primary visual cortex.

Blindsight, as opposed to residual seeing in which the viewer acknowledges having a visual percept, was first described³ in 1973 and has never been free of sceptics. The objections have been various — that it is an artefact of scattered light, of poor control of the subject's eye movements or of using a lax criterion for the subject's guesses, or that it stems from the activity of remnants of striate cortex (or a combi-

nation of these). Each has been satisfactorily rebutted in many instances.

Another 'objection', which amounts to dismissing its oddness and therefore its interest, is that blindsight is just like normal vision near the threshold for detection⁴, where it has long been known by psychophysicists that normal subjects perform statistically better than expected by guessing even when they are uncertain about seeing anything⁵. Although a dissociation between detecting and localizing faint visual stimuli has been demonstrated in normal subjects, who localized at statistically significant levels despite failing to detect better than expected from guessing⁶, Kolb and Braun point out that this dissociation may be restricted to performance and not awareness. Hence their new experiment.

Their observers looked at a VDU, on which a display like that shown in the figure appeared briefly, and had to indicate in which of the four quadrants the target appeared — that is, guessing should yield only 25 per cent correct overall. In the actual displays the target square was gen-



A display like that used by Kolb and Braun¹, showing how a target area can be defined by a simple change in orientation of lines on the screen.

erated by different directions of motion of the dots within the target and the surround, or by orthogonal orientation of line elements. Furthermore, in each condition the targets could be made either easy to see (awareness) or impossible to see (unawareness), by means of retinal rivalry in which the eyes are given conflicting information or by adjusting the separation of pairs of moving dots. But the novel feature of the procedure was to ask subjects after every trial how confident

MATERIALS SCIENCE

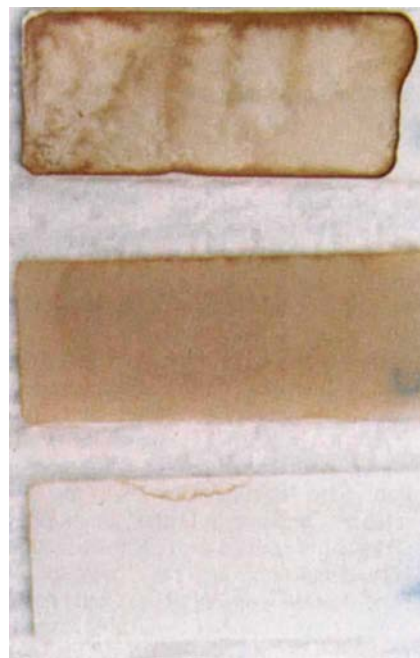
Window, clean thyself!

WINDOW cleaners, your days are numbered. Self-cleaning window glass is on the horizon, as described by Adam Heller of the University of Texas, Austin, at a meeting of the American Chemical Society*. A photocatalytic coating of titanium dioxide just 60 nanometres thick on ordinary soda-lime glass will photo-oxidize 200 nanometres per day of organic contaminants in normal daylight.

The photocatalytic properties of TiO_2 have been recognized for decades, and have been explored for diverse applications ranging from oil-spill remediation to artificial photosynthesis. Moreover, photographic lenses are routinely coated with TiO_2 to convey improved toughness and antireflectivity. But a layer of the oxide deposited onto ordinary window glass is not photochemically active, because the free-radical-induced oxidation reaction is suppressed by the diffusion of sodium from the glass into the TiO_2 film.

Heller has shown that pretreating soda-lime glass with boiling concentrated sulphuric acid for 30 minutes will leach out the labile sodium ions. A TiO_2 thin film can then be applied by spin-coating a titanium alkoxide precursor onto the

glass, hydrolysing and calcining at 500 °C. To extend this treatment to the indus-



Clean gone — top to bottom, coffee stains on ordinary latex paint, on a photocatalytic coating kept in the dark, and on the same coating after exposure to sunlight. (Courtesy E. Heller & Co., California.)

trial scale, Heller believes that it would be necessary to introduce the sodium-leaching stage into the glass processing, before the molten glass is formed into sheets. A car windscreen made in this way might incur an additional cost of perhaps \$50–100 to the customer.

A self-cleaning windscreen would encounter another problem, however: because of the relatively high refractive index of the TiO_2 coating, the coated glass would be highly reflective, so that a tilted windscreen would contain a prominent reflection of the dashboard. It might then be necessary to reduce the reflectivity of the dashboard itself, for example by applying a polarizing polymer film (light reflected from dashboards is generally polarized, and so can be suppressed by a cross-polarized film).

The TiO_2 films prepared in this way are clear and polycrystalline. But the idea might be extended to the opaque titania emulsions used in white paint, leading to self-cleaning coatings that can be painted onto any surface. The Toto company in Japan is developing such paints, and has just begun to market photocatalytic ceramic tiles for use in hospitals and bathrooms, whose photocatalytic action will be driven by the ultraviolet component of fluorescent strip lighting.

Philip Ball

* National Meeting of the American Chemical Society, Chicago, 20–24 August 1995.

they were about their response, on a scale from 1 to 10. In this way the experimenters were able to correlate performance and subjective experience quantitatively.

The results were both clear and surprising. With the displays in which the target was designed to produce perceptual awareness, the fraction of correct responses correlated well with the confidence rating — that is, when observers were confident about being correct they were correct. However, when using the displays where subjects reported no perceptual awareness, there was no correlation between confidence rating and performance. This difference occurred despite the fact that overall performance on the conditions of awareness and non-awareness was similar and high (about 70–80 per cent correct). In other words, one can perform well above, and not just above, threshold yet be perceptually unaware of what it is one is responding to.

Why is this demonstration important? First, it introduces confidence judgements on every trial, enabling performance to be tightly related to subjective experience. Trial-by-trial binary judgements (Yes/No) about awareness were recently introduced to demonstrate that unawareness is compatible with excellent discrimination in blindsight⁷, but the new procedure should allow a much finer grained analysis within the unaware mode. Second, it shows that the dissociation between performance and awareness is neither a phenomenon restricted to vision near threshold, nor to blindsight. Third, it cautions against attempts to use blindsight as a model for rehabilitation by attempting to train observers to use unacknowledged visual stimuli in their blind fields. When lacking awareness, even practised subjects could not gauge anything cognitively useful about the visual displays. It seems that methods of rehabilitation in blindsight patients will require the restoration of awareness in order to be effective.

Finally, and provocatively, Kolb and Braun's results suggest that as the kinds of displays where the observer is neither aware of the target nor confident about his guesses are known to be relatively poor at stimulating cells in certain extrastriate visual areas, the latter might be the normal 'site' of visual awareness. This could explain why, when striate cortex is

destroyed and the main input to extrastriate cortex removed, the patient is unaware of most visual stimuli despite performing well; and why damage confined to extrastriate visual cortex can also produce clinically blind field defects⁸.

Many of us will want to know whether the new results are more easily explained by the possibility that 'invisible' targets can nevertheless trigger eye movements that could be used to guide 'guesses' about target location. But this is testable, as are predictions, again based on proper-

ties of cells in extrastriate visual areas, about other kinds of targets (generated by colour, or luminance or retinal disparity) that can be correctly responded to with or without awareness. The success of Kolb and Braun will be assessed in a few years by how many investigators are aware or unaware of their intriguing demonstration. □

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LOW-TEMPERATURE PHYSICS

Atom bouncers have it taped

Wayne M. Itano

EXPERIMENTERS now have a new set of tricks for manipulating and storing cold atoms — and the key ingredients are familiar high-street commodities. Edward Hinds and co-workers at Yale University have repeatedly bounced rubidium atoms from magnetic tape of the kind used to record audio signals¹. In later experiments, they obtained better results with floppy disks.

A particle that has a magnetic moment is attracted to or repelled from a region of high magnetic field, depending on which way the magnetic moment is pointing. So a localized region of high magnetic field reflects slow particles, provided that their magnetic moments are first oriented in the proper direction. As far back as the 1920s, Stern and Gerlach used spatially non-uniform magnetic fields to deflect the trajectories of silver atoms, each of which has a magnetic moment due to an unpaired electron. But the deflection produced was slight. Normal-incidence reflection, as observed by the Yale group, required methods for creating a strongly varying magnetic field as well as methods for slowing the atoms before attempting to reflect them.

The arrangement of magnetic fields used in the recent reflection experiments was discussed in 1961 by Vladimirskii². The application he had in mind was the production of focused beams of polarized neutrons (that is, neutrons that have their spins and hence their magnetic moments oriented in the same direction). He pointed out that a surface on which the magnetic field reverses direction periodically as one moves parallel to the surface along one direction (say the x direction) and which is constant as one moves parallel to the surface along the perpendicular (y) direction would form a magnetic field suitable for a plane magnetic mirror. Away from the surface, the magnitude of the field decreases exponentially, as $|B| = B_0 \exp(-kz)$. Here B_0 is the magnitude of the field at the surface, k is 2π divided

by the spatial period of the surface magnetization, and z is the distance from the surface.

The direction of the field changes rapidly, but the orientation of the magnetic moment with respect to the field direction will be preserved if the particle moves slowly enough. The magnetic force will then depend only on the magnitude

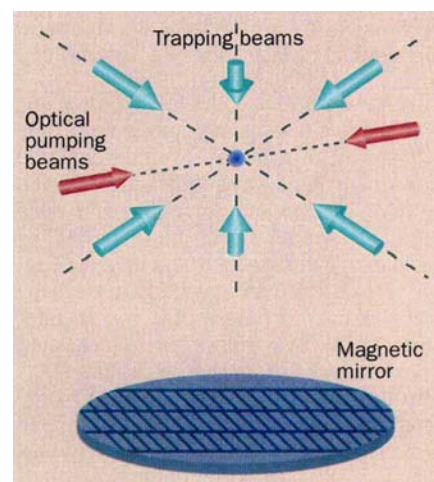


FIG. 1 A magneto-optic trap, made from a combination of laser beams and magnetic fields, collects and cools a sample of rubidium atoms, which are released and allowed to fall. The optical pumping beams orient the magnetic moments of the atoms so that they will be repelled from the magnetic mirror, which is made from audio cassette tape or a floppy disk. After Fig. 1 of ref. 1.

of the field, not its direction. As the particle approaches the surface, it will be subjected to an exponentially increasing magnetic force. If it is going slowly enough and if its magnetic moment is pointing in the right direction to begin with, it will be reflected.

Vladimirskii originally proposed creating such a plane of alternating magnetic field by laying down an array of parallel conductors in which the direction of the

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electrical current alternated from one to the next. More recently, Opat, Wark and Cimmino³ pointed out that a better method was to record a suitable magnetization pattern on a ferromagnetic substrate, such as audio recording tape.

Atoms have previously been reflected at normal incidence from evanescent light fields. An optical electric field, tuned above an atomic resonance, causes the charge distribution within the atom to oscillate at the same frequency, but with a phase lag. This generates a positive interaction energy, so that an atom is pushed

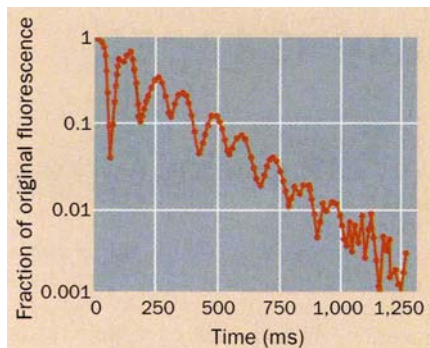


FIG. 2 Fraction of atoms recaptured in the magneto-optic trap as a function of the time after being dropped onto a concave magnetic mirror made from a floppy disk. At least 8 bounces can be seen. After Fig. 2 of ref. 6.

away from regions of high light intensity. If the light is totally reflected at the inside surface of a transparent medium, the evanescent light wave that leaks out can form an atomic mirror. The first such 'atomic trampoline' was made in 1990 by Kasevich, Weiss and Chu⁴, who saw sodium atoms bounce twice after being dropped from a height of 2 cm. In later experiments by Aminoff *et al.*, caesium atoms bounced more than eight times⁵.

In the new experiment reported by the Yale group, a magnetic mirror was created by recording a 5-kHz audio tone on cassette tape with an ordinary tape recorder. The spatial period of the magnetization was 9.5 μm . Three strips of tape were cut out and glued to a 2.5-cm flat glass substrate to form the mirror. The field just outside the tape is 0.11 tesla, which would be strong enough to reflect rubidium atoms dropped from 0.8 m, although the atoms were actually dropped only a few centimetres.

The experimental arrangement is sketched in Fig. 1. Rubidium atoms are collected in a magneto-optic trap, consisting of a non-uniform magnetic field and three pairs of mutually perpendicular laser beams, detuned below resonance. After enough atoms have accumulated, the frequencies of the lasers are further detuned from resonance, which cools the atoms to about 20 microkelvins. The laser beams and magnetic fields that make up the trap are then shut off, allowing the

atoms to drop onto the magnetized tape. Before the atoms fall out of the trap region, they are optically pumped by two of the laser beams so that their magnetic moments have the proper orientation to be reflected. The optical pumping has the side-effect of heating the atoms to 30 microkelvins. After a variable time, the magneto-optic trap is switched back on, and the number of atoms retrapped is recorded.

Multiple bounces were observed with this apparatus, for a duration of 600 milliseconds. Only 63 per cent of the atoms fell on a magnetized region of the tape, but, of those that did, about 94 per cent were reflected. The reflection was specular. The reflectivity of demagnetized tape was also high, but was diffuse, presumably because the random orientations of the magnetic domains create a magnetic mirror that has a rough surface.

An improved version of this experiment uses a pattern with a 10- μm period recorded on the surface of a floppy disk. A sine-wave audio signal was sent directly to the recording head, bypassing the ordinary recording electronics. The stepper motor of the drive mechanism was modified so that successive tracks overlapped, and the pattern was recorded over the entire available surface. A 2.5-cm diameter circular piece was cut out and stretched into a concave shape, which focused the reflected atoms and allowed more bounces to be seen. Figure 2 shows the fraction of the atoms recaptured as a function of the time after they are released — bounces can be clearly seen out to about 1 second.

These magnetic methods for reflecting atoms are certainly simpler to implement than optical methods, but will they prove as useful? One remaining question is whether coherence can be preserved in the reflection. If so, magnetic mirrors could be used as retroreflectors in atom interferometers. If not, there might still be applications that do not require coherence, such as storing atoms, free from laser radiation, for use in some other experiment. We could imagine creating a magnetic 'bucket of atoms', perhaps containing some rare isotope. This would give a double meaning to the term 'magnetic storage medium'.

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DAEDALUS

Oil for ever!

LAST WEEK Daedalus decided that some marine catalyst must be catalysing the production of crude oil and oxygen from sea water and its dissolved carbon dioxide. This would explain the strangely low concentration of CO_2 in air and sea; and the oceanic oil slicks which tanker operators ascribe to natural geological seepage. All catalysts, even oxidation catalysts, must be reversible. This deep-ocean oxidation catalyst works in reverse because the hydrocarbon oil rises to the surface as fast as it is formed, perturbing the catalytic equilibrium towards making more of it. It may be some fairly widespread marine mineral or molluscan shell surface.

Daedalus now wants to tap this steady oceanic upwelling of oil. The great ocean currents will sweep it along; where they sink downwards for their undersea return journeys, the floating oil will be unable to follow, and will concentrate at the surface. The main downwelling areas are off Greenland in the Northern Hemisphere and in the Weddell Sea in the Antarctic. They are too big to sweep for oil directly. Cunningly, Daedalus will do it by means of a strategically positioned and anchored iceberg.

He points out that the ocean can divide spontaneously into convection cells, in which warm water rises and cool water sinks. The plume of cold water sinking down from an iceberg could initiate and maintain a stable convection cell covering a huge area. Water will then sweep towards the berg from all points of the cell, bearing the oil with it. More cunning still, cold water has a higher surface tension than warmer water. The colder inner region of the cell will draw the oil inwards even faster by Marangoni convection — the pull of differential surface tensions. A gutter round the iceberg will scoop up the oil as it floods in, and pumps will deliver it to storage containers on the berg. Daedalus likes the idea of using the iceberg as an oil tanker, and towing it and its cargo to the nearest feasible port; but it will probably be better to transfer the oil at intervals to conventional vessels. Any given iceberg will melt in a few months. Another will then be chosen from nearby candidates, and towed into position. The operation will run continuously.

Our fuel problems will then be over. Marine catalytic oil synthesis, ultimately powered by solar and geothermal heat, will supply endless oil for fuel and feedstock. The CO_2 from its combustion will not accumulate in the atmospheric greenhouse, but will dissolve in the seas to be recycled back to more oil. Gas-guzzlers will be free to guzzle serenely on.

David Jones

The aspidistra and the amphipod

SIR — The aspidistra is an aberrant member of Convallariaceae (Monocotyledon: Asparagales)¹, which bears bell-shaped flowers close to the ground and was thought to be unique in being pollinated by slugs². The pollination system of the aspidistra, however, has not yet been directly reported in its original area of distribution of East Asia. Recent observations made in its native habitat in Japan suggested that *Aspidistra elatior* is pollinated by pollen-eating, terrestrial amphipods.

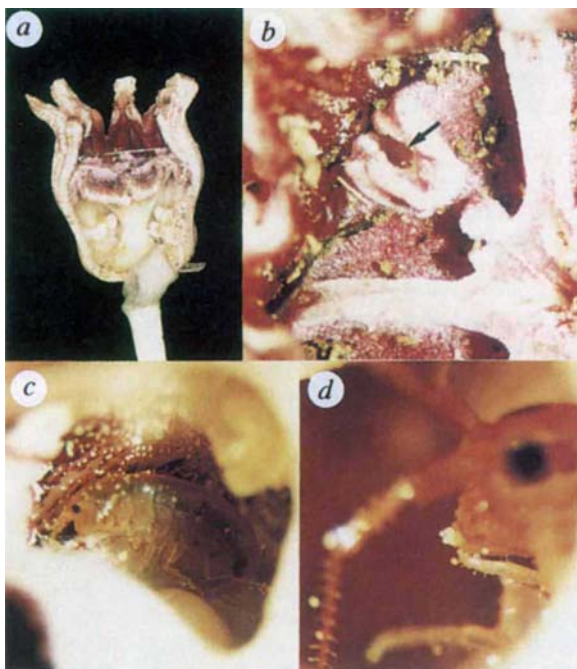
The most unique characteristic of the aspidistra is a large, fleshy, disk-like stigma, which entirely blocks the lower inside corolla containing the stamens (*a, b* in the figure). The stigma of *Aspidistra lurida* is believed to act as an attractant and reward for slugs, which partly eat the stigma, gaining access to the stamens below, and thus taking part in pollination².

Aspidistra elatior Bl. is widely cultivated in China and Japan, but is thought to be indigenous to only a few small islands in the southern part of Japan³. The higher altitudes (300–622 m) of the island of Kuroshima in Kagoshima Prefecture are covered with evergreen oak forests whose understory is covered with aspidistras. We made observations of pollinator visits to the flowers here in March 1995.

No flowers were found with stigma damage. Among 89 flowers sampled and dissected, 37% were visited by various arthropods; isopods (8%), amphipods (8%) (*c* in the figure), collembolans (14%), thysanurans (1%), dipterans (3%, predaceous larvae of Mycetophilidae) and chiropods (2%). In 28% of the flowers, yellowish-white faeces composed of digested pollen were present, but no visitors were found. In these flowers, almost all pollen grains had disappeared. Pollen would accumulate in the bottom of the flower if not

consumed by an organism.

To detect which organisms had left these faeces, five arthropod species (*Sphaerillo* sp. (Crustacea: Isopoda: Armadillidae), *Burmoniscus* sp. (Isopoda: Philosciidae), *Platorchestia japonica* (Amphipoda: Talitridae), *Hypogastrura* sp. (Insecta: Collembola: Hypogastruridae) and *Scolopocryptops* sp. (Chilopoda: Cryptopidae)) most frequently found in aspidistra flowers were observed in the laboratory. These arthropods, collected in or around the flowers, were introduced in plastic cases with aspidistra flowers which had been cut transversely. Among arthro-



A flower of *Aspidistra elatior* and its amphipod pollinator, *Platorchestia japonica*. *a*, Vertical section; *b*, top view of a stigma. An arrow shows a pore through which amphipods enter below. *c*, Vertical section of a flower being visited by an amphipod; *d*, an amphipod feeding on pollen.

pods studied, amphipods and collembolans ate pollen (*d* in the figure), and amphipods excreted faeces which were identical with those left in the flowers in their natural habitat. The only slug species found on the islands, *Granulilimax fuscicornis* (Mollusca: Pulmonata), preyed on snails and never visited the flower.

Because anthers of *Aspidistra* are isolated from the upper disk-like stigma, self-pollination is unlikely if the flower is not visited by an organism. Seed-set confirmed in the natural habitats and the evidence of frequent amphipod visits to flowers suggest that the amphipod is the most likely candidate for the pollinator.

The pollinator status of the amphipod is further reinforced by the following evidence. (1) Between the disk-like stigma and the corolla, there are four small,

narrow pores, through which the amphipods gained access to the stamen (*b* in the figure). The stigma acted as a block to rain and other flower-damaging arthropods, and as a gate for the selected pollinators. (2) The amphipods visited the flower to eat pollen, and left the flower with pollen attached to the body. (3) The amphipods cannot fly but are very able hoppers, and could thus transport pollen long distances.

Although the terrestrial talitrid amphipods are thought to have originated in Gondwanaland and reached South-East Asia on drifting land fragments^{4,5}, the Japanese species are thought to have adapted to terrestrial habitats rather recently from supralittoral habitats^{6,7}. To detect whether the unique pollination system is ubiquitous amongst *Aspidistra*, observations are needed in the area of its greatest diversity, the southern parts of China.

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Cortical areas in visual awareness

SIR — I commend Crick and Koch¹ for their serious and innovative neurobiological approach to the problem of visual awareness and agree with several of their ideas, such as those for differential explicit representations from cortex to cortex. Space, however, constrains me to confine subsequent comments largely to areas of disagreement.

Crick and Koch hypothesize that “activity in V1 does not directly enter awareness”. I feel that they should clarify an ambiguity in their text and their Fig. 1: do they believe that frontal cortical areas are involved in visual awareness, and if so, why? I also challenge their premise that only those visual areas that project directly to brain regions that “contemplate, plan and execute voluntary motor outputs” can participate directly in awareness. I disagree with claims that existing physiological and psychophysical evidence supports their hypothesis, and offer here evidence against excluding V1 and V2 from the process of visual awareness.

Neural correlates of working spatial and object memory are found in premotor cortex². Ablations of frontal cortex do not affect visual acuity but may produce ‘neglect’, a stimulus–response failure in the absence of a sensory defect, attributed to “attentional components of motor responses, and not elementary, space-related visual perception”³. If Crick and Koch believe that these cortices and/or premotor and motor cortex are involved in visual awareness, I ask them to provide such evidence.

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Alternatively, if they do not believe that frontal cortex is directly involved in visual awareness, their requirement for a direct connection to frontal areas from visual cortices involved in awareness loses much, though not all, of its relevance. Suppose that awareness of elemental form, though not of its symbolic content or identification, depends on neuronal activity, either directly in V1 or more likely as part of dynamic iterative interactions between V1 and higher cortices. Then, as long as adequate intermediary neural traces, perhaps distributed over multiple extrastriate areas, are conveyed to 'decision-making' regions, I see no *a priori* necessity for neurons in perceptual space to communicate directly with those in decision space.

Although the evidence that (partial) colour constancy requires the activity of neurons in V4, this result does not prove that neurons involved in achromatic form perception do not reside in V1 or V2. Crick and Koch attach much weight to the report of He *et al.*⁴ that certain high-contrast, very high-spatial-frequency gratings, although invisible, produce an orientation-selective loss in sensitivity to gratings of slightly lower spatial frequency. He *et al.* used interference fringes to obviate the diffraction limits of the pupil and display gratings of high spatial frequencies never before seen by their subjects. These stimuli presumably activate at least some neurons in V1, but they may be wired only to inhibit other neurons selective for slightly lower spatial frequencies. Alternatively, the mature brain may not construct an unambiguous percept for isolated presentations of such ultra-high-spatial frequencies never experienced during normal visual development.

Crick and Koch's premise that "the relevant aspect of a message must be explicitly coded or one cannot see that aspect" does not preclude a direct involvement of V1 and V2 in awareness. For example, brightness is explicitly represented as early as V1⁵ and specific 'illusory' contours correlate with neural activity either in V1⁶ or V2⁷.

An explicit representation of form may exist in cortical areas lower in the cortical hierarchy than those subserving coherent motion and colour discrimination. Human form vision survives injury to cortical area MT even when the motion signal is lost⁸. Even when colours appear washed out or are lost in central achromatopsia from lesions beyond the striate cortex, perception for varying shades of grey remains intact. The brain-injured achromatopsic artist described by Sacks and Wasserman could distinguish letters and claimed his vision "became that of an eagle. The sharpness of focus is incredible. But — I am absolutely colour blind."³

Lesions beyond V4 do not produce achromatic field deficits. Although it was assumed for almost a century that the

most distal lesions in the human visual hierarchy causing static achromatic visual-field defects were located in V1, Horton and Hoyt⁹ have convincingly shown that lesions involving V2/V3 — even with V1 intact — may be sufficient to create a visual-field defect. "Because V1 and V2/V3 are arranged in a serial fashion, a lesion in V2/V3 is functionally equivalent to a lesion in V1, ignoring for a moment the projection from V1 to V5."⁹ This suggests that V2 and V3 cannot be excluded on anatomical grounds from circuits involved in visual awareness, and neither can V1, as the adjacent extrastriate lesions have disconnected V1 from higher cortical areas. These results do suggest that the apparently intact connections from V1 to subcortical areas such as the superior colliculus, the pulvinar, the lateral geniculate nucleus and claustrum are not sufficient to subserve visual awareness.

I do not claim that visual awareness for any perceptual attribute must reside in V1, V2 or V3, either alone or collectively by means of their feedforward and feedback interconnections. The proximal extrastriate cortices project both subcortically and to widespread regions of temporal and parietal cortex. Moreover, V1 and V2 are targets of 'back projections' from several extrastriate, temporal and limbic associated cortices^{10–12}. Dynamic iterative interactions between 'recognition spaces' and 'perceptual spaces' may well be a minimal requirement for visual awareness.

Crick and Koch rightly suggest that awareness may require construction of a multilevel explicit symbolic interpretation of parts of a visual scene involving different levels in the visual hierarchy. The above considerations do, however, argue against excluding V1, V2, and perhaps even V3, from this hierarchy. Finally, I urge Crick and Koch to suggest a testable experiment that could disprove their hypothesis.

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CRICK AND KOCH REPLY — An unstated assumption in our thinking¹ is that, in terms of neural 'computation', we make a distinction between the computations

themselves and their results. A rough analogy would be between all the activity within a computer (the computations) and the results of those computations expressed on the screen. We are aware of some of the results, but are largely unaware of the computations themselves.

This tentative assumption is supported by experiments showing that, in a binocular rivalry task, only a minority of the neurons in cortical area MT that respond to the visual input follow the percept reported by the monkey¹³. Moreover, these neurons are now known to be located in the two lower layers of the cortex¹⁴. We believe that some of these neurons may form a special subset which "express the results of the computations." It is important to find out where such neurons project. Contrary to received opinion, it may be simpler to find out how the results of the computations are expressed (the neural correlate of consciousness) than to find out exactly how the very elaborate computations are done.

To deal with Pollen's specific comments: We are suggesting that the different aspects of visual awareness are explicitly expressed in various visual cortical areas, but that a person is not conscious of them unless the relevant neurons project to some part of the motor planning system, which we tentatively identified as located in parts of the premotor and/or prefrontal cortices. It is a myth that total ablation of the human frontal cortex does not affect awareness in visual acuity tests. We know of no case in which a person has lost the whole prefrontal and premotor cortex, on both sides (including Broca's area), and can still see.

Most of Pollen's comments seem to us irrelevant. To disprove our hypothesis one has to show that there are at least some neurons in V1 whose firing correlates with some aspects of what is seen and that there are no neurons in higher cortical areas whose firing correlates with exactly the same aspect of the visual scene. In other words, that there are neurons unique to V1 that correspond to part of what we actually see. Pollen has not shown this.

To prove our hypothesis, it would be necessary to record from every type of neuron in V1 that expresses properties unique to V1 and show their firing correlates with aspects of the visual field of which we are unaware. We have said¹ that

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it would be difficult to prove our ideas by such methods, giving as a single example the presumed absence of the Land effect in V1 neurons. Pollen is correct in pointing out that this argument deals with only one or a few types of neurons in V1. It may also be true that the very high spatial frequencies that we cannot see⁴ may excite solely inhibitory cells in V1, though we doubt this. It might be possible to test this by recording from alert macaque monkeys, but it would not be easy.

We are grateful to Pollen for drawing our attention to the work of Horton and Hoyt⁹. This shows rather clearly that for a patient with a quadrantic visual field defect, who is blind within that part of the visual field, the relevant portion of V1 is largely, if not entirely, intact.

This result might initially seem to support our hypothesis very strongly. However, we agree with Pollen that although V1 is structurally intact, it is not receiving all its usual inputs, especially those coming back from V2 and V3, because the relevant parts of these regions are damaged. If these back-pathways are essential for visual awareness, Horton and Hoyt's observations would not support our hypothesis; however, Pollen gives no evidence that a person would be totally blind without these particular back-pathways. Until the exact impact of such pathways is determined experimentally, the matter must remain open. If these

pathways (in a macaque or lower mammal) were knocked out, and the animal could still see, then cases similar to those reported by Horton and Hoyt, provided there was no subcortical damage, would strongly support our hypothesis.

We agree with Pollen that if the connections from (surviving) V1 to subcortical areas were intact in such patients, the results would show that those connections are not, by themselves, sufficient to subserve visual awareness. Of course, it may be that, despite this, one or more of these connections is necessary for visual awareness.

Several papers, both on blindsight in normal observers¹⁵ and the McCollough effect in patients who cannot see orientation^{16,17}, are compatible with the idea that striate cortex activity by itself can influence behaviour without producing awareness, but this evidence is not strong enough to prove our hypothesis directly.

In summary, we regard our hypothesis as plausible but at present difficult to prove. Pollen may not like our ideas, but he has produced no evidence that decisively disproves them.

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The amygdala and emotional memory

SIR—Animal studies indicate that memory storage for events that arouse emotions involves neurobiological processes consisting, at their simplest, of activation of β -adrenergic receptors and the amygdaloid complex¹. Recently, we reported that in normal human subjects, β -adrenergic blockade selectively impaired long-term (1 week) memory for an emotionally arousing short story². We report here parallel results from a patient with impaired function of the amygdaloid complex.

The patient (B.P.) suffers from Urbach-Wiethe disease, an extremely rare hereditary disorder that has produced bilateral brain damage confined to the amygdaloid complex region (Fig. 1), with no evidence of the seizures seen in some Urbach-Wiethe patients³. Recent psychological tests show B.P. to be in the normal range for many cognitive functions, including atten-

tion, intelligence and short-term memory⁴.

In the present investigation, B.P. was told a short story, presented as a brief, narrated slide show, about a young boy walking with

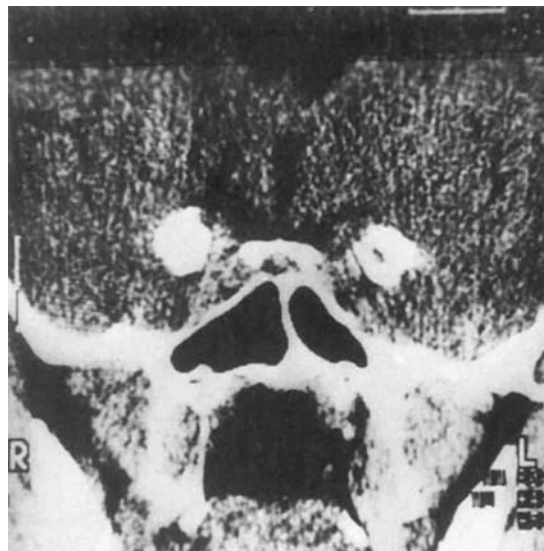


FIG. 1 Coronal computer tomography scan showing bilateral mineralization in the amygdaloid complex in patient B.P. Additional brain-scan images have been reported elsewhere⁴. (Photograph courtesy of Neurological University-Clinic, Knappschaftskrankenhaus Bochum, Germany.)

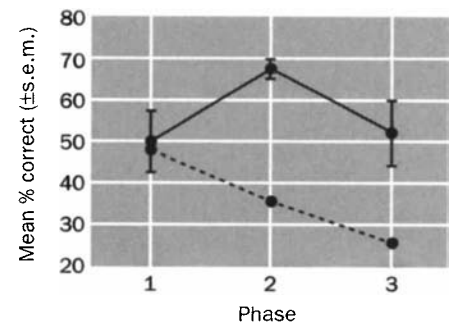


FIG. 2 Memory test performance for controls (solid line; $n=6$) and patient B.P. (dotted line). Controls were matched to B.P. for age (mean age, 34.8 ± 1.5 yr; B.P. age, 37 yr) and for education (mean number of school years, 10.5 ± 0.67 ; B.P., 10 yr). Controls show the expected increase in memory for phase 2 of the story, in which the strong emotional elements were introduced (phase 3 is the final phase of the story).

his mother to visit his father at work. The story was slightly modified from that used in our previous report (for example, it was translated into German), but retained the essential features of the original version. At one phase of the story, emotional events (involving severe injuries to the boy and graphic pictures after he is involved in a terrible traffic accident) are introduced.

Consistent with our previous report, results from the German control subjects indicate that memory for this phase of the story (phase 2) is consistently superior to that of the relatively unemotional initial story phase 1 (as assessed with a multiple-choice recognition test one week later; Fig. 2). In sharp contrast, B.P. showed no evidence of enhanced memory for phase 2 of the story despite normal memory for story phase 1. B.P.'s self-assessed emotional reaction to the story (determined immediately after viewing the story) was similar to that of the controls: mean emotional rating of the story (on a scale of 0 to 10) for controls was 7.25 ± 1.2 ; B.P. rated his reaction to the story at 8. Thus, B.P. failed to show the normal increase in memory associated with emotional arousal despite a normal self-assessed emotional reaction to the story.

These results are consistent with earlier findings from this and another Urbach-Wiethe patient indicating selective impairment of memory for emotional material^{4,5}. The results may be related to demonstrations of impaired recognition of emotion in faces by patients with lesions of the amygdaloid complex^{6,7}, as well as to the

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established role of this brain region in conditioning of autonomic processes during emotional learning situations^{8,9}.

Considered together with our previous study², these findings support the view that the influence of emotional arousal on conscious, long-term memory in humans involves β -adrenergic receptor activation and influences mediated by the amygdaloid complex. Furthermore these processes seem not to be required for normal retention in non-emotionally arousing circumstances.

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A methanotrophic carnivorous sponge

SIR—Associations between methanotrophic bacteria and several species of mytilids and a pogonophore have been described in deep-sea communities surrounding hydrothermal vents and cold seeps¹. We report here a new symbiosis between a sponge and methanotrophic bacteria. The $\delta^{13}\text{C}$ values and distribution indicate that the sponge is nutritionally reliant on its methanotrophic symbionts. Ultrastructural evidence indicates intracellular digestion of the bacteria. Intracellular symbionts were found in brooded embryos, indicating direct transmission of the symbionts between generations.

The sponge, *Cladorhiza* sp., belongs to a unique deep-sea family, Cladorhizidae (Demospongiae), which are carnivorous, and lack an aquiferous system². The specimens were collected from a mud volcano in the Barbados Trench at a depth of 4,943 m. Large aggregations of hundreds of individuals (up to 1.5 m in diameter and 40 cm high) were found around the periphery of the volcano's eye, where the highest concentrations of methane were documented

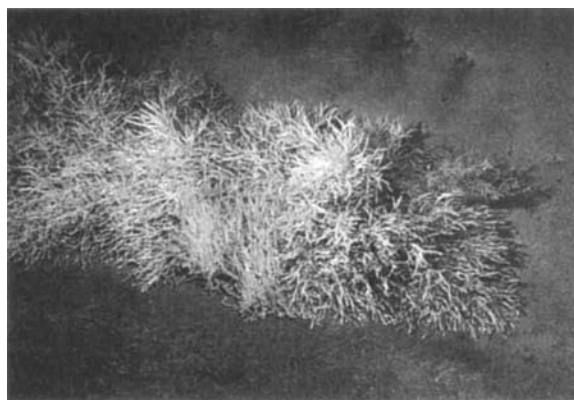


FIG. 1 Large bush of cladorhizid sponge observed with the submersible *Nautilie* at 4,943 m in the mud volcano Atalante, in the Barbados Trench, during the MANON cruise. The sponges were most abundant and larger around the volcano eye where large amounts of methane were detected.

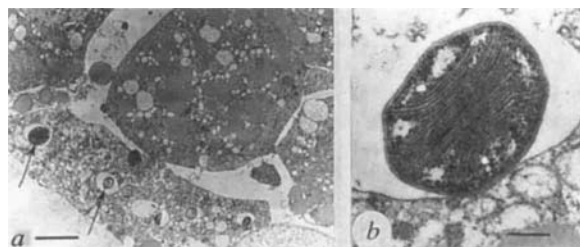


FIG. 2 Transmission electron micrographs of microsymbionts within the surface cells of the embryo. *a*, Two bacterial types (arrows). Scale bar, 2.7 μm . *b*, Magnification of a methanotrophic symbiont with stacked lamellae. Unlike bacteria inside somatic cells, there is no evidence of bacterial degradation in the embryo cells. Scale bar, 0.3 μm .

ed³ (Fig. 1). Before this discovery, cladorhizids were known to occur only in low densities as discrete individuals.

Two ultrastructural types of symbiotic bacteria were observed in the sponge tissue (Fig. 2). (1) Coccoid cells with stacked intracytoplasmic membranes and electron-lucent droplets. This morphology is typical of some methanotrophic bacteria, including methanotrophic symbionts described in other invertebrates. (2) Smaller, oval-to-rod-shaped cells. Both symbionts occurred intercellularly, apparently healthy, with frequent division stages, and intracellularly in phagocytic cells in various stages of degradation, suggesting a nutritional relationship between host and symbionts.

Many symbionts were also observed within flattened cells at the surface of embryos. In this location, no degenerating symbionts were apparent. This suggests a mechanism for direct transmission of the symbionts between generations and is reminiscent of littoral 'bacteriosponges', where bacteria are transmitted between generations in oocytes.

Sponge tissues were tested for the presence of key enzymes of methylotrophic and chemoautotrophic sulphur-oxidizing bacteria. Significant methanol dehydrogenase activity was found, indicating the presence of abundant methanotrophic symbionts. Low activities

of ribulose 1,5-bisphosphate (RuBP) carboxylase were detected, which could be due to type x methanotrophs, low activity in chemoautotrophic symbionts, or surface bacteria. Neither ATP sulphurylase nor adenosine-5'-phosphosulphonate reductase activities were detectable.

The very negative tissue $\delta^{13}\text{C}$ values (from -48.4 to -48.8‰ in three samples) are also consistent with the presence of methanotrophic symbionts. The relatively light tissue $\delta^{15}\text{N}$ values (+2.0 to 2.9‰) are within the range reported for other vent and seep fauna, and reflect significant input of organic nitrogen of local origin⁴.

These observations indicate that the symbionts are methanotrophic and suggest that the sponge derives some of its nutrition from the intracellular digestion of the symbionts. A similar mechanism has been suggested for a variety of other chemoautotrophic and methanotrophic symbioses¹, as well as for littoral 'bacteriosponges' with heterotrophic symbionts⁵.

The sponge morphology, erect with branching processes bearing a cover of hook-like spicules, suggests they may also feed on swimming prey, as do other cladorhizids⁶. This was supported by the presence of debris from small crustaceans on the sponges. The relative importance of carnivory and methanotrophy in sponge nutrition cannot be evaluated, but it is unlikely that such aggregations of carnivorous sponges could survive in the deep sea without a nutritional symbiosis.

Carnivory has allowed cladorhizid sponges to colonize the deepest oceans, although they are normally small and rare. The association with methanotrophic bacteria, with their unusual feeding strategy, allows a large biomass, and illustrates the extreme adaptability of *Porifera*.

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The killing fields

J. R. S. Fincham

The Last Harvest: The Genetic Gamble that Threatens to Destroy American Agriculture. By Paul Raeburn. *Simon and Schuster: 1995. Pp. 269. \$24.*

SUCCESSFUL plant breeding depends on a pool of genetic variation to which selection can be applied. But by producing highly productive varieties that take over large sectors of the market, its natural tendency is to drive much of that variation out of existence. At the same time, the reservoir of wild species from which cultivated plants were derived and could in principle be regenerated is constantly being diminished by commercial development and population pressure. There has to be concern about the ability of plant breeders to meet future crises, whether brought about by new pests and diseases or by climatic change.

Paul Raeburn provides a highly readable survey of these issues for the layman. He is a journalist rather than a scientist, but he has consulted and quotes a broad range of scientific authorities, including representatives of seed companies as well as academic botanists and ecologists. He wants to raise the alarm, but nevertheless his book is not the apocalyptic tract that its catchpenny title and subtitle would lead one to expect. The careful reader can come away with a broad and balanced picture of the problem.

There are two ways in which genetic diversity for future breeding can be conserved: through seed banks and through conservation of wild plants in their natural habitats. The United States, on which this book is mainly focused, has large publicly funded seed banks, including the National Seed Storage Laboratory, said to hold about a quarter of a million samples of different species and cultivars, and the National Small Grains Collection, which houses 112,000 different accessions of all the commonly grown cereals, including rice.

Raeburn highlights the difficulties and hazards in the maintenance of such extensive collections. The policy at the National Seed Storage Laboratory is to grow and regenerate more seed from any sample whose viability drops below 65 per cent, but it emerged from Raeburn's enquiries that nearly two-thirds of the collection had never been tested for its ability to germinate, either because of lack of staff or because the numbers of seeds were too small to risk in a test. Ideally, all these small samples should be grown into plants for generation of more adequate quantities of seed but, given the general difficulty of getting funding for things whose usefulness has not yet

been demonstrated, one cannot be surprised that this has not been done. The evaluation of the agronomic potential of each and every seed sample would obviously be a very much greater job, yet without such evaluation the collection is not readily usable. Directors of seed banks have to do the best they can with limited resources, and they may well be haunted by Raeburn's chapter heading: "Seed Banks and Seed Morgues".

Regarding the preservation of species in the wild, there are some success stories but not enough. Raeburn cites the Sierra

some exaggeration, on its "almost magical ability to shuffle genes as easily as a deck of cards", but he assumes that desirable genes for incorporation into crops would have to come ready-made from nature rather than by *in vitro* reconstruction based on knowledge of how they work. This may well be to underrate the power of genetic engineering, but at least one can agree that it is never likely to make natural variation redundant.

How imminent is the crisis of genetic impoverishment foreseen in Raeburn's book? The examples that he cites from the recent history of US agriculture are as much of problems overcome as of disasters. The infestation in the late 1980s of the wheat and barley crop by Russian aphid was countered, though with much effort and over many years, by the development of resistant varieties incorporating genes from several Middle Eastern strains available in the National Small

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Crops in crisis: Recent cases of genetic impoverishment in US agriculture are examples of problems overcome rather than disasters.

de Manantlan Biosphere Reserve, run by the University of Guadalajara in Mexico, which is home to upwards of 2,500 plant species, including a perennial maize. But the establishment of such reserves around the world will depend not only on the political stability of the countries concerned but also on successful diplomacy. Most of the world's plant species, including nearly all of those still to be discovered, are in poor countries increasingly inclined to demand a price for the exploitation, or even the exploration, of their native floras. Raeburn recounts how Thomas Jefferson enriched his country's agriculture by smuggling rice seeds out of Piedmont in Italy, thereby risking execution by the Piedmontese. Though no doubt praiseworthy at the time, this kind of botanical piracy can hardly be defended today.

Raeburn's position on genetic engineering is ambivalent. He remarks, with

Seeds Collection. Such examples might well suggest to the optimist that plant breeders have genetic resources sufficient to keep agriculture going, at least for the time being. But, in the longer term, radical changes in growing conditions brought about, for example, by soil deterioration and all the consequences of global warming if and when it occurs, could force changes both in methods of cultivation and in the kinds of plants grown. Raeburn, in his final chapter, advocates a return to smaller-scale (and presumably more labour-intensive) farming, with a greater diversity of crops.

The key to the maintenance of genetic diversity is, of course, money. How much can we afford? □

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Mission science

Roy Gibson

Launching Europe: An Ethnography of European Cooperation in Space Science.

By Stacia E. Zabusky. Princeton University Press: 1995. Pp. 261. \$49.50, £37.50 (hbk); \$17.95, £14.95 (pbk).

International Cooperation in Space: The Example of the European Space Agency.

By Roger M. Bonnet and Vittorio Manno. Harvard University Press: 1994. Pp. 163. \$39.95, £24.95.

THE title of Stacia Zabusky's *Launching Europe* and its cover illustration of a computer-designed satellite obscure the threat contained in the subtitle: *An Ethnography of European Cooperation in Space*. Readers can safely traverse the book's pages

ingenuity in inventing a case for new allowances, but a request for danger money would really be breaking new ground. Nor would the author's hosts be likely to share her view that the practice of working together on a scientific satellite project is considered a "sacred duty".

The anthropologist Raymond Firth, of 'malay Fishermen' fame, taught that such ethnographic studies should aim to help outsiders to interact more effectively (and less intrusively) with the group being studied and also help at least some of the members of the group to understand how they might most easily improve their lot. Zabusky's book does neither, and it is a relief to turn to one that really does cover the ground its title announces: *International Cooperation in Space*. Roger Bonnet has for some years been the science director at ESA, and as such is directly responsible for the space-science department.

He is a well known and respected scientist, with a long involvement with European scientific cooperation. Vittorio Manno is largely a product of ESA and its predecessor, the European Space Research Organization, and has several decades of experience both as a practising space scientist and in the corridors of space-science power.

The outcome of their cooperation is a very readable look at how European space scientists started their successful collaboration, not only between scientific groups in Western Europe, but also with scientists in North America, Russia, Japan and a string of other countries. Their first-hand account of the political and financial (and occasionally scientific) vicissitudes — as seen from Europe — is as

accurate and unbiased as one might expect from two such distinguished scientists.

The book is, however, less successful when it moves beyond space science and attempts to continue its analysis into the realms of application satellites and manned space flight. These newer activities in space — telecommunications, meteorology and the rest — all certainly owe their existence and rapid development to the persistence and perspicacity of the space-science pioneers, but the overall space-related problems that now face governments and space agencies in many parts of the world have outgrown the original space-science mould. Whether or not one likes it — and the authors understandably do not — decisions on space policy and development

involve factors that go far beyond the competence of space agencies. Increasingly, politics, economics, industrial policy and other 'non-space' matters have greater weight than purely space considerations. It is not surprising, therefore, that Bonnet and Manno's analysis of this wider subject is less satisfying than the chapters on space science, where their obvious competence shines through.

International Cooperation in Science is nonetheless a book to read and even to buy. It makes a particularly effective antidote to the toxic effects that Zabusky's book might produce on sensitive souls such as this reviewer. □

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Cure thyself

Anthony Storr

Cultures of Healing: Correcting the Image of American Mental Health Care.

By Robert T. Fancher. W. H. Freeman: 1995. Pp. 355. \$23.95, £16.95 (hbk).

"THE distance between what we know and what we wish we knew is too great to bear, and we fill it with believing," Robert T. Fancher, who has a PhD in philosophy and who has worked in public-policy analysis, became disillusioned with both. Philosophy seemed to him arid, and the so-called 'scientific studies' on which public policy-making was based turned out to be promoting particular ideologies rather than being objective. Fancher then trained as a psychotherapist and practises in New York city. He has brought his critical, sceptical eye to bear on the assumptions about human nature that underlie the practice of psychotherapy. In doing so, he has written a stimulating and controversial book.

Fancher's concept of 'mental health care' is limited. Although he has chapters on the history of psychiatry, he is little concerned with contemporary treatment of the major psychoses such as schizophrenia and manic-depressive illness and omits any consideration of mental handicap, Alzheimer's disease, drug-related problems, alcoholism or any of the many other disorders requiring specialized psychiatric treatment or care in institutions. For Fancher, modern 'mental health care' is psychotherapy. Today, on both sides of the Atlantic, many thousands of people seek help from psychotherapists because they are distressed and have 'problems in living'. Different schools of psychotherapy each claim validity. Fancher's concern is with examining the unacknowledged premises supporting these claims. The

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High hopes: ESA's first Ariane 4 rocket just before its launch in 1988.

without fear of increasing their understanding of the problems of 'launching' either European space or European space science. A few readers will perhaps be either sufficiently knowledgeable or sufficiently charitable to regard the book as a useful contribution to anthropology, but to those familiar with the space-science community, the mixture of melodrama and mysticism does not ring true. "Working on the edge of death is simultaneously exciting and terrifying", concludes the author, after nearly a year observing the workings of the space-science department at the European Space Agency's technical establishment in the Netherlands. This may well be true, but it is grotesquely out of place in a study of the space-science community. ESA staff have never lacked

problems raised are philosophical rather than scientific, but he still hopes for a psychopathology based on science.

Fancher affirms that it was the Second World War that in the United States gave a huge impetus to the idea of mental illness, because so many potential recruits to the armed forces were rejected as mentally unfit. In addition, "around 850,000 soldiers were diagnosed with mental problems". The war encouraged the proliferation of mental-health professionals. It also wrongly encouraged the belief that such 'experts' could define the boundaries between mental health and illness. Psychoanalysis became the dominant 'culture' in psychiatry, partly because of the promotional effectiveness of Karl and William Menninger, who founded the Menninger Clinic and School of Psychiatry based on Freudian ideas, and partly because of the influx of psychoanalysts who fled Europe to escape Hitler. In the 1950s it was impossible to gain a senior position in psychiatry in the United States unless one had undergone psychoanalytic training. Today the opposite is the case. Freud has been so discredited within psychiatry that psychiatrists in training in the United States know nothing about him. The rise and fall of psychoanalysis is a fascinating chapter in social history. Fancher is of course right in dismissing Freud's claim to be a scientist, but this is nothing new. I think he is also right in writing: "The greatest legacy of psychoanalysis is its highly developed art of listening for what is not being said, for self-deception that is being perpetrated, and for the wishes and terrors that patients cannot own honestly."

At the opposite pole to psychoanalysis is behaviour therapy, supposedly based on sound psychological principles that are ultimately derived from Pavlov. Hans Eysenck, the leading critic of psychoanalysis in the United Kingdom, promoted behaviour therapy as a scientifically based treatment for a variety of psychological problems supposed to be the pathological result of faulty conditioning. Behaviourists believe that whatever has been learned can be unlearned. Some of the techniques employed by behaviour therapists have undoubtedly been effective in a very limited field; but, as Fancher demonstrates, behaviour therapy has nothing to offer many of the patients seeking psychotherapy. B. F. Skinner of Harvard, one of the most famous behaviourists of modern times, wished to abolish "the man defended by the literature of freedom and dignity" and wrote that a "scientific analysis of behavior dispossesses autonomous man and turns the control he has been said to exercise over to the environment". The behaviourist picture of human nature is quite inadequate.

Fancher devotes a fair amount of space to Beck's 'cognitive therapy', which is

based on the hopeful notion that if you can alter the ways in which people think about themselves they will become better adjusted. It is indeed the case that people who are prone to depression see the world through dark glasses, expecting the worst, denigrating themselves and reinforcing their pessimistic outlook by taking account only of events and opinions that fit in with it. But, as Fancher points out, many depressives have long histories of failure at love and work and have failed to get what they want from life. "In the last fifteen years, a substantial body of data has accumulated showing that depressives are actually much more accurate than nondepressed persons in their appraisal of themselves and their circumstances."

Biological psychiatry assumes that mental illness arises from malfunction of the brain and is largely genetically determined. Clinical appraisal of a case of depression in a modern psychiatric outpatient clinic will generally revolve around two questions. First, which label from the *Diagnostic and Statistical Manual of Mental Disorders* fits the case in question? Second, which is the best drug to use to lift

the depression? Fancher is as scathing about the inadequacy of the assumptions underlying biological psychiatry as he is about all other psychiatric methods.

Yet here he is, practising as a psychotherapist. How does he justify it? Outcome studies have demonstrated that between about two-thirds and four-fifths of patients benefit from therapy, whatever the method employed. In considering verbal therapies, Fancher does not make enough of the uniqueness of the psychotherapeutic situation. In what other personal encounter do people have the opportunity to talk about themselves at infinite length without fear of interruption or rejection? Perhaps simply airing one's difficulties, and expressing one's miseries, makes them easier to cope with? One of my teachers said that he had treated a patient who visited three times a week for a year. During the whole of that time the therapist said nothing whatsoever. At the end of the year the grateful patient pronounced himself cured. □

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Hyping up the letdown gene

Mike Stratton

Breakthrough: The Quest to Isolate the Gene for Hereditary Breast Cancer. By Kevin Davies and Michael White. *Macmillan: 1995. Pp. 370. £16.99. To be published in the United States by Wiley.*

THE race to isolate the gene for susceptibility to breast cancer (*BRCA1*) was one of the most publicized medical stories of the past few years. Huge media and public interest was generated by the fact that breast cancer is a tragically common disease which most people have encountered at some time or another through their family or friends. Along the way, this interest was stoked up by frequent tantalizing rumours that the gene had been isolated, by the distinctive personalities of many of the main characters in the plot and by the many complex accessory issues, particularly the potential for commercial exploitation of the gene sequence.

Kevin Davies and Michael White tell the *BRCA1* story and use it as a starting point for a wide trawl through the epidemiology, psychology, sociology and politics of breast cancer. They chart the way in which the disease has become an important political issue in the United States. Development of breast cancer by prominent American women (particularly presidents' wives) contributed to a transformation in public awareness that provided a new focus for the American women's movement after its many successes in

re-orienting society's attitudes in the workplace. The emergence of professional lobby groups with expertise in media management converted '1 in 9' from a statistic (the proportion of women in the United States who develop breast cancer) to a powerful recriminative chant against the lack of success (and, some would charge, lack of effort) of the medical and scientific establishments in fighting breast cancer.

Non-American readers may find this a strange and rather fascinating landscape. Some scientists will remain sceptical about whether public pressure for advancement necessarily influences progress on intractable problems of the natural world (and will be able to quote precedents). The authors generally refrain from comment, conducting a usually balanced argument in the form of quotations from the parties involved. But this call for change has undeniably channelled more resources into breast-cancer research, and the authors illustrate the way in which an increasing number of scientific and medical issues have ceased to be the private concern of health professionals but have become subject to the consumer demands of a vocal and critical American marketplace.

Breakthrough is informative and meticulously documented. But it also aspires to be a dramatic narrative written in a popular literary style that conveys the excitement and tension of the period and describes the fluctuating fortunes in the

race. To my mind, however, there is a flaw in the plot. Isolation of *BRCA1* was an extraordinary technical *tour de force*, a major medical advance and perhaps the last great all-out battle of the big gene-cloning empires. Yet when the sequence was released, there was a palpable sense of anticlimax, a feeling that comes through in the book. It was as if the culprit in a crime novel turned out to be someone who nobody had heard of and who lacked any obvious motive. There were few surprises, the principal one being the rather disappointing discovery that *BRCA1* does not seem to be frequently involved in common, sporadic breast cancer. Moreover, it became clear that *BRCA1* is not the only culprit and that at least one more breast-cancer gene is involved. So the isolation of *BRCA1* recedes into what it always was: an important step in a progressive increase in knowledge about a terrible disease. A breakthrough — certainly. The Breakthrough — perhaps not.

Breakthrough is part of a wave of books that caters to the increasing public interest in science and medicine. Some of the more detailed accounts of the gene mapping and medical management may be hard going for the general reader and the more hard-headed may wish to skip the potted biographies of the main protagonists. Nevertheless, as an all-embracing account of the current state of breast cancer, the book has something for everyone, from the previously uninformed reader to the breast-cancer specialist. □

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Children's Books

Nature plans to publish on 16 November a supplement in which children's books and software will be reviewed. The supplement will cover new publications for children of all ages.

Publishers are invited to send suitable material for consideration, taking note of the following criteria:

- Only books and software issued in 1995 will be considered;
- Books and software dealing with any aspect of science, technology, medicine, natural history or the environment are eligible (including encyclopaedias, dictionaries and games), although school curricula texts are excluded;
- The main language used must be English;
- If possible, cross-platform software (both Macintosh and PC) should be provided.

Publications for review should be sent immediately, together with details of price and availability, to Peter Tallack, Book Review Editor, *Nature*, 4 Crinan Street, London N1 9XW, UK (tel: +44 (0)171 843 4567; fax: +44 (0)171 843 4596/7; e-mail: p.tallack@nature.com).

Science in the dock

John Buckleton

Science and the Detective: Selected Reading in Forensic Science. By Brian H. Kaye. VCH: 1995. Pp. 388. DM68 (pbk); DM148 (hbk).

Interpreting Evidence: Evaluating Forensic Science in the Courtroom. By Bernard Robertson and G. A. Vignaux. Wiley: 1995. Pp.240. £24.95.

FORENSIC science is a broad topic, and modern books on the subject are typically highly specialized, usually written by several different authors. Not so *Science and the Detective*. The author, a physicist with an interest in lexicography, aims to interest the lay public as well as the legal community and other concerned professionals.

Although the volume is studded with fascinating case anecdotes, these are often

fusingly entitled "Foot prints". For good reasons, however, practitioners in the field deliberately make a distinction between the two kinds of prints: one connects to a shoe, the other to a suspect.

The book is also remarkably behind the times. There is a description of old methods of glass examination but no mention of the modern temperature-variation methods widely used since the 1970s; in the short section on DNA, only the obsolete multilocus technique is illustrated; and for all the suspense, readers do not even learn the answer to the Anna Anderson mystery, although the solution has recently been published.

By contrast, *Interpreting Evidence* will appeal to both lawyers and forensic scientists. It has a serious message and is not light reading. The authors are prominent workers in the fascinating yet neglected field of forensic interpretation. This, their first book on the subject, is an important attempt to bring clarity to a confusing area.

The authors rightly contend that lawyers and forensic scientists must communicate in the same language if miscarriages of justice are to be avoided. The stumbling-block lies, they argue, in the lack of understanding of Bayesian inference, first outlined in 1763 but brought to the attention of the forensic community in the 1970s and 1980s by workers such as Ian Evett and Dennis Lindley. This philosophy is now applied widely by the forensic community in parts of Europe and New Zealand yet largely ignored in the United States and Australia.

Bayesian inference follows from simple laws of probability and directs the tutored mind to ask the relevant questions, in this case with respect to legal trials but also more generally in all decision-making. Even in countries applying the approach, there is considerable scope for improvement in both scientists' and lawyers' understanding of Bayesian logic.

Forensic evidence, such as DNA, can provide answers to such questions as: "What is the probability of this DNA match if the sample did not come from the suspect?" But courts are more interested in questions such as: "What is the probability that this DNA sample came from the suspect?" The authors demonstrate why these are different questions and explain how to proceed from one to the other.

The book contains many actual case examples such as the O. J. Simpson and the Birmingham Six trials, all worked through in such a way that the authors' points are irrefutable. One must hope that their work is widely read and understood by everyone to whom forensic evidence is important. □

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REASONS

Hands on forensics: technicians check DNA fingerprints.

described only briefly, and what details there are often betray a lack of a proper understanding of the subject. The numerous definitions of terms, on the other hand, are tiresomely lengthy and painfully precise. The word 'autopsy', for instance, is traced back to its Greek roots and is then immediately followed by the definition of 'biopsy' in a section almost as long as the discussion of whether Anna Anderson was indeed Anastasia, as she claimed. In particular, the aspects of forensics involving physics are in many cases over-emphasized. The discussion of fingerprints, for example, has a long section on laser luminescent enhancement whereas the uniqueness of fingerprints is naively accepted in just a couple of paragraphs, with scant regard paid to the quality of the prints. There is merely passing mention of the concept of a point, with reference to the 16-point rule only, and little discussion of the relevance of such rules, despite the fact that they constitute the largest single field in forensic science.

The book cannot even be used as a 'forensic dictionary': it is not set out for this purpose, nor is it accurate enough. For instance, the section on shoe prints is con-

The role of mantle plumes in continental breakup: case histories from Gondwanaland

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After thirty years of plate-tectonic theory, the reasons why supercontinents disintegrate and disperse to form smaller continental plates remain enigmatic. Possible causes range from abnormally hot mantle upwellings, or plumes, to changes in plate-boundary driving forces. The breakup of the Gondwanaland supercontinent, which started about 180 million years ago, provides an excellent case history against which to test models.

THROUGHOUT geological time, continents have drifted over the Earth's surface as rigid plates¹. Periodically, they have collided and aggregated to form large supercontinents, and large supercontinents have disintegrated to form smaller plates. Before 180 Myr ago, today's southern continents, together with India, were grouped to form a large continent referred to as Gondwanaland (Fig. 1). The breakup and dispersal of a supercontinent like Gondwanaland² represents a radical change in the plate-tectonic regime, and the reasons for this dramatic change are hotly debated³. Current ideas involve either the plate and boundary forces that drive plate motions or some active internal mantle process⁴ (Fig. 2).

Most workers assume that when the continents are dispersed as they are today, the plates either drive themselves, where passive subsidence of cooling oceanic plates causes them to move away from mid-ocean spreading ridges and to be consumed (subducted) eventually at destructive plate boundaries (Fig. 2)⁵⁻⁷, or that the plates are carried by frictional drag forces exerted by the internal mantle convecting system⁸. However, many workers doubt whether these forces are sufficient to cause a supercontinent to break up, and consequently invoke an active-mantle mechanism involving an abnormally hot upwelling in the mantle known as a mantle plume or hotspot⁹⁻¹³ (Fig. 2). Plumes are generally envisaged as having a narrow central conduit arising from a thermal disturbance at the core-mantle boundary with, at least initially, a large bulbous mushroom head up to 2,000 km in diameter^{14,15}. They are considered to be an important mode of mantle convection that is independent of plate-scale mantle flow. Larson¹⁶ speculated that there may have been major periods in Earth's history when larger mantle plumes (for example, in mid-Cretaceous times (~120 Myr) in the present-day southwest Pacific Ocean), termed superplumes, drastically affected both the pattern of mantle convection and plate motions.

A common factor of mantle-plume models is that plumes have the capacity to generate large quantities of basaltic magma^{14,15,17} often before continental breakup (for example, continental flood basalts), and to produce chains of volcanic islands as oceanic plates move over fixed hotspots, but the major issue of relevance here is their role in initiating continental breakup. Ideas vary from the original active mantle hypothesis of Morgan^{10,11}, who considered that plumes drive the plates and initiate continental breakup by doming and cracking the continents, and pushing the continents apart, to passive models, where the plume plays no part in breakup except that the chance unroofing of a pre-existing or incubating plume results only in rapid outpouring of flood basalt provinces¹⁴. Some hybrid models combine aspects of both active and passive models: the forces that drive plate motions place the continents under tension when subduction is taking place on both sides of a continent but it is the arrival of

a new plume that weakens them and causes them to split and form a new ocean¹⁸⁻²⁰.

Although plumes are generally considered as representing a thermal disturbance at the core-mantle boundary¹⁵ (Fig. 2), Anderson²¹ suggested that hot regions could develop at shallow levels in the mantle owing to the insulating effect of supercontinents, and that the build-up of heat beneath a supercontinent could lead initially to thermal expansion and horizontal temperature gradients in the mantle. This could be followed by doming and fragmentation of the continent, and ultimately to the dispersal of continental fragments from hot to cold mantle regions^{22,23}.

The present upsurge of interest in mantle dynamics, the development of new mantle-plume hypotheses and ensuing controversies have led to a re-evaluation of their role in the disintegration of large supercontinents²⁴. The testing of geological models can come only from the natural laboratory, and the fragmentation of Gondwanaland into smaller continents² (Fig. 1) provides an excellent case history against which to test various models. From a study of Gondwanaland it seems that, from currently available evidence, plumes are not the ultimate driving force for continental breakup; it can proceed without a plume, and can generally be linked to changes in plate-boundary forces and in the overall plate-tectonic regime. When mantle plumes are involved, they may have assisted breakup and controlled its location. By contrast, mantle plumes may be the dominant cause of the splitting of smaller continental blocks.

Stages in the breakup of Gondwanaland

There were three main episodes in the disintegration of the Gondwanaland continent² (Figs 1 and 3). The initial rifting stage started in Early Jurassic times (~180 Myr ago), and led to a seaway (Fig. 1b) forming between West (South America and Africa) and East Gondwanaland (Antarctica, Australia, India and New Zealand), and to sea-floor spreading in the Somali, Mozambique and possibly Weddell Sea basins where the oldest known sea-floor magnetic anomalies are M22 (~156 Myr)^{25,26}. The second stage occurred in Early Cretaceous times (~130 Myr) when this two-plate system was replaced by three, with South America separating from an African-Indian plate, and the African-Indian plate from Antarctica (Fig. 1c). Finally, in Late Cretaceous times (90–100 Myr), the breakup of that once large continent was completed when Australia and New Zealand separated from the Antarctic core, and other small continental blocks, Madagascar and the Seychelles separated from India as it migrated northwards away from Africa and Antarctica (Fig. 1d).

Continental separation was in most cases closely associated in time with extrusion of flood-basalt provinces that are generally linked to mantle plumes and in some cases connected by

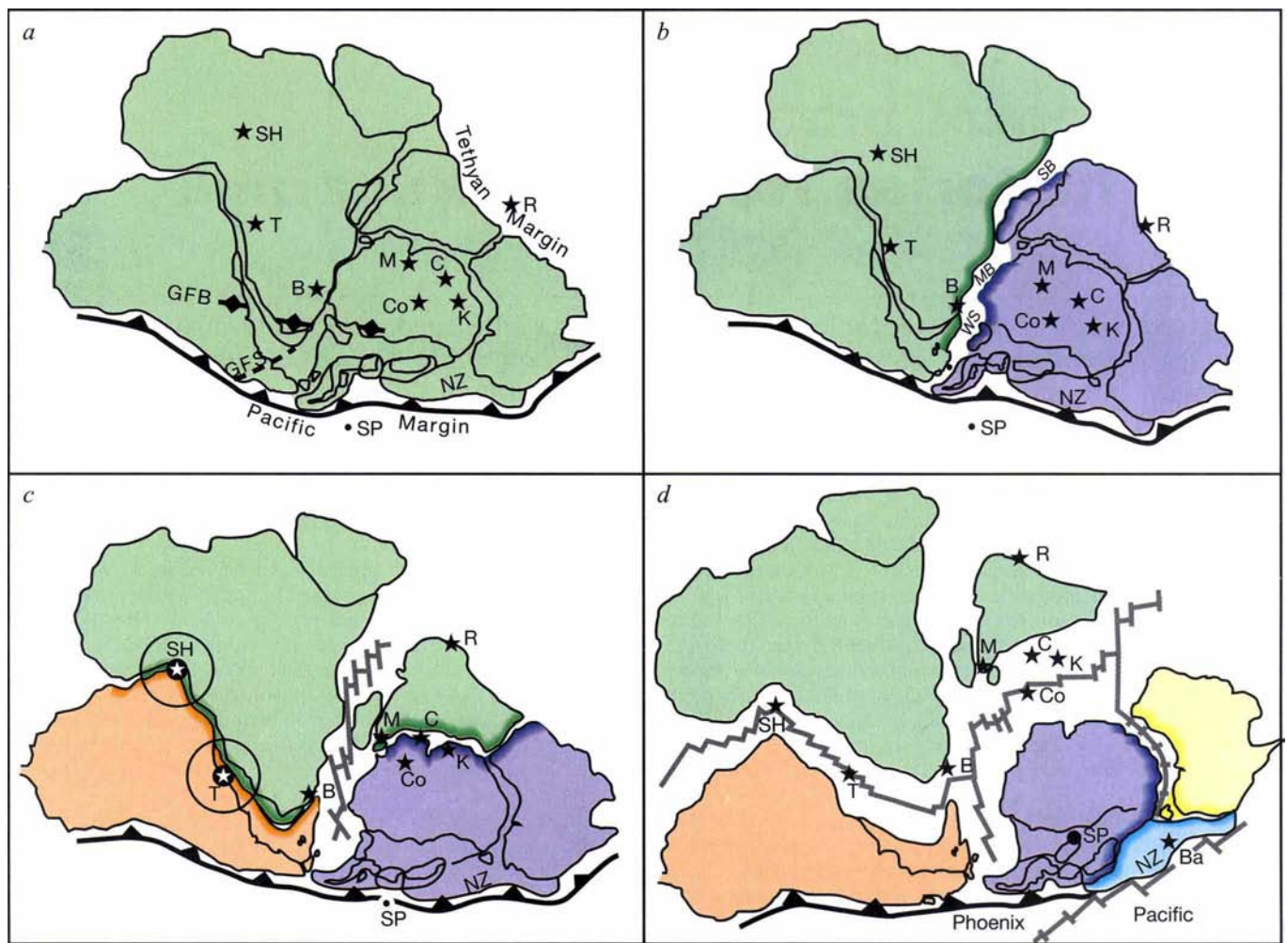
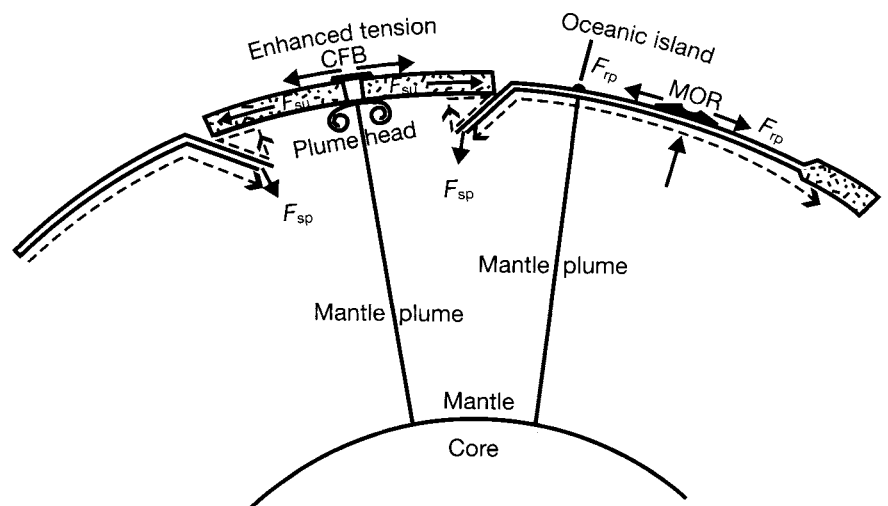


FIG. 1 Gondwanaland reconstructions for (a) 200 Myr, (b) 160 Myr (c) 130 Myr and (d) 100 Myr based on Lawver *et al.*². The reconstructions show the main plates that existed within each time frame (different colour for each plate), the active rifted margins (darker colours), the position of the main subduction zones, and the position of present-day mantle hotspots using the Gondwanaland coordinates in Lawver *et al.*² based on the assumption that mantle hotspot positions are fixed relative to one another through time. Note how the Indian Ocean hotspots (C, Co, K and M) were located beneath the thick East Antarctic craton at 200 and 160 Myr. The time at which hotspots produced massive

volumes of basaltic magma is shown with a partly filled symbol and a circle showing the size of a postulated 2,000-km plume head¹⁴. Abbreviations for plumes are as follows: Ba, Balleny; B, Bouvet; C, Crozet; Co, Conrad; K, Kerguelen; M, Marion; R, Réunion; SH, St Helena; T, Tristan da Cunha; GFS, Gastre Fault System; GFB, Gondwanian fold belt; MB, Mozambique basin; NZ, New Zealand continental block; SB, Somali basin; SP, South Pole; WS, proto-Weddell Sea. The oceanic spreading ridges are diagrammatic, illustrating approximate boundaries of the major plates.

FIG. 2 Diagrammatic cross-section of the Earth's crust showing the main forces that operate on continental and oceanic plates^{3,6}, and mantle plumes arising from the core-mantle boundary. F_{rp} , ridge push forces; F_{sp} , subduction slab-pull forces; F_{su} , trench suction forces; CFB, continental flood basalt; MOR, mid-ocean ridge. The diagram assumes that plumes originate at the core-mantle boundary.



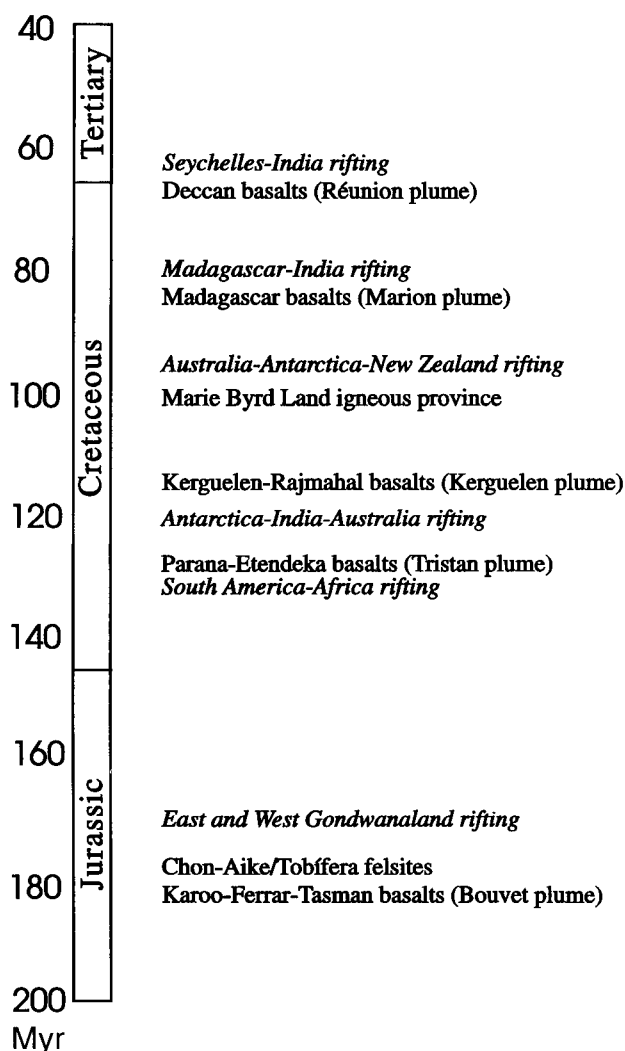


FIG. 3 Summary of main Gondwanaland breakup events.

chains of volcanic islands to known present-day hotspots¹⁷ (Fig. 4). However, in the Indian Ocean, there is some debate (discussed below) on the correlation of specific hotspots and basalt provinces before ~120 Myr because of uncertainties in Gondwanaland plate motions relative to the fixed hotspot reference frame and the closeness of some hotspots^{27,28}.

Initial rifting stage

Before and during the initial rifting stage, the onland geological record indicates that the Gondwanaland continent was bounded, on the Pacific side, by an active subducting margin²⁹ (Fig. 1a). As no ocean floor older than the Jurassic survives in the Pacific basin, we are not aware of its plate configuration at this time, but by Late Jurassic times a three-plate system existed, involving the Pacific, Farallon and Phoenix plates, with subduction of the Phoenix plate along the Gondwanaland margin³⁰. On the Tethyan side (Fig. 1a), the margin changed from a subducting to a passive margin in Triassic times after rifting of the Cimmerian continent (parts of present-day Turkey, Afghanistan, Iran, Tibet, China and Indo-China) from Gondwanaland and opening of the Neotethyan Ocean³¹.

The initial rifting stage followed a period of coeval compressional and extensional deformation of Gondwanaland³². The most dramatic effect of the compressional deformation was the formation of the Triassic Gondwanian fold belt (Fig. 1a) that included the Cape and Sierra de la Ventana fold belts of

southern Africa and South America, respectively, and their southerly continuation seen in the Ellsworth and Pensacola mountains in Antarctica. As well as subduction, the early stages of rifting were contemporaneous with formation of a large within-plate magmatic province at least part of which, the Karoo province in southern Africa³³, was related to a mantle plume³⁴ beneath the Gondwanian fold belt. The significance of this coincidence and the role of the mantle plume and subduction process during breakup is uncertain and is discussed below after a brief description of the within-plate magmatic province and the active Pacific margin of Gondwanaland.

Within-plate magmatic province. The large magmatic province within Gondwanaland formed a long linear belt that stretched from southern Africa (Karoo province³³) through Antarctica (Ferrar province³⁵) to Australia (Tasman province³⁶) (Fig. 5). A connection between the Karoo province in southern Africa and a mantle plume³⁴ is consistent with the volume and composition of the volcanic rocks³³ and the uplift history of southern Africa³⁷. Morgan¹¹ suggested that the Crozet hotspot could have lain beneath the province in Jurassic times, whereas owing to uncertainties in plate motions, Duncan and Richards¹⁷ and Lawver *et al.*² favoured the Marion and Bouvet hotspots respectively. Although two major episodes of basaltic volcanism at 193 ± 5 Myr and 178 ± 5 Myr (refs 33, 38) have previously been recognized within the Karoo province and have led to a two-stage rifting model³⁹, new ⁴⁰Ar–³⁹Ar dating from a 1,500-m section indicates that the basalts were erupted during a short-lived event at 182 ± 2 Myr (ref. 40). The main phases of Antarctic Ferrar and Tasmanian rift-related magmatism have been dated at 176.6 ± 1.8 Myr (ref. 41) and 175 ± 18 Myr (ref. 36) respectively, close to the new age of the Karoo province. The new ⁴⁰Ar–³⁹Ar dating of part of the Ferrar province⁴¹ indicates an eruption history within a short interval of less than 1 Myr in common with many other flood-basalt provinces in the world.

The linear extent of the Gondwanaland province for upwards of 5,000 km, subparallel to the palaeo-Pacific margin of Gondwanaland (Fig. 5), is not compatible with conventional circle-shaped models of mantle plumes. Moreover, Brewer *et al.*⁴² have identified a geochemical transition within the Transantarctic Mountains between rocks that have a chemical composition consistent with a plume source, to rocks of the Ferrar province that do not. Although the volume of the Ferrar rocks, estimated to be 5×10^5 km³ (ref. 43) is relatively small compared with many flood basalt provinces, it is still large enough to suggest that it was produced above a mantle with higher-than-normal temperatures. Cox⁴⁴ preferred to replace the notion of a hotspot by a hot-line. He suggested that the main melting anomaly responsible for the province was related in some way to subduction, and drew analogies between the province as a whole and back-arc spreading.

The within-plate province is not entirely restricted in composition to basaltic rocks, and contemporaneous granitic rocks within West Antarctica are considered to be genetically part of it²⁹. Furthermore, in southern South America, one of the largest silicic volcanic provinces in the world (the Chon-Aike or Tobífera province)⁴⁵ was contemporaneous, and in a reconstructed Gondwanaland virtually continuous with, the Karoo-Ferrar-Tasman basalt province⁴⁶ (Fig. 5). The silicic province is associated with extensional grabens that first formed in Triassic times⁴⁷ in central Patagonia, and with a major Early Jurassic dextral shear zone, termed the Gastre Fault System⁴⁸, that dissected southern South America.

New isotopic dating of Jurassic volcanic rocks in Patagonia to the north (Marifil Complex) and south (Chon-Aike or Tobífera) of the Gastre Fault System suggest a short-lived (3–5 Myr) and well-defined igneous event (180 Myr) derived from an isotopically uniform source for at least part of this province⁴⁹. Various mechanisms have been proposed for production of this uniform province including crustal melting⁴⁵, fractionation of an enriched Ferrar-type mantle source⁴⁶ and rapid remelting of

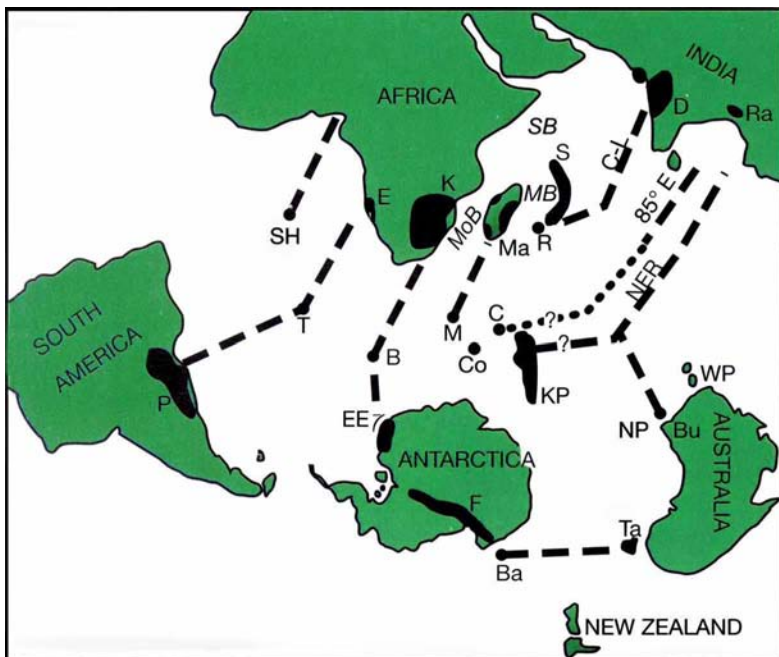


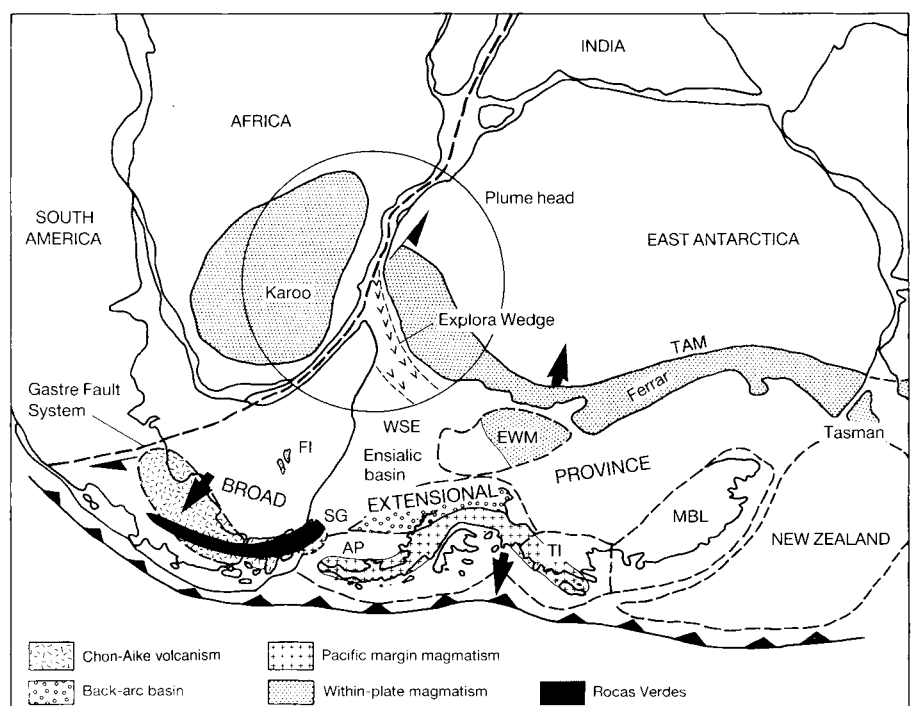
FIG. 4 Present-day map of the former Gondwanaland continents illustrating the most likely connection between flood basalts (Bu, Bunbury; D, Deccan; E, Etendeka; EE Explora Escarpment (formerly a wedge of seaward-dipping volcanic rocks); F, Ferrar; K, Karoo; KP, Kerguelen Plateau; Ma, Madagascar; NP, Naturaliste Plateau; P, Parana; Ra, Rajmahal; Ta, Tasmanian; WP, Wallaby Plateau) and known hotspots (see Fig. 1 for abbreviations). C-L, Chagos-Laccadive; MB, Mascarene basin; MoB, Mozambique basin; NER, Ninetyeast Ridge; SB, Somali basin; S, Seychelles.

underplated mafic rock⁴⁹. However, irrespective of their petrogenesis, the important point here is that the silicic province represented a period of high heat flow and magma production over an extensive region of Gondwanaland contemporaneous with the Karoo-Ferrar-Tasman magmatism. This could have been produced by a number of different mechanisms including channelling of the Bouvet mantle plume from beneath the Karoo province to neighbouring regions of thinner crust, by a mantle plume situated closer to the silicic province than the Bouvet plume (for example, Shona plume 500 km west of Bouvet^{50,51}), by a mantle plume with a more irregular shape than the usually envisaged circular shape, or by tapping a hotter-than-normal mantle layer beneath the extending lithosphere. The difference between the largely silicic Chon-Aike, and the largely mafic Karoo-Ferrar-Tasman provinces may have been simply a func-

tion of the magmatic plumbing system; in the mafic province, primary magmas may have reached the surface quickly, whereas in the silicic province, mafic magmas may have underplated beneath the crust and either fractionated to form the silicic rocks or caused crustal melting of the surrounding lower-crustal material.

Rift basins. In Late Jurassic times (150 Myr), broadly coincident with the earliest known age of sea-floor spreading in the Mozambique and Somali basins and marking the beginning of Gondwanaland dispersal, the extensive Chon-Aike silicic province was split by the formation of a rift basin⁵². The basin is now represented by uplifted ophiolitic rocks known as Rocas Verdes in southern South America⁵² and the Larsen Harbour complex in their southerly continuation in South Georgia⁵³ (Fig. 5). Lavas associated with initial rifting have elemental and isotopic signatures

FIG. 5 Antarctic sector of Gondwanaland illustrating the main Middle Jurassic magmatic provinces²⁹ and the location of the Bouvet plume¹⁴, ~180 Myr. AP, Antarctic Peninsula; EWM, Ellsworth-Whitmore Mountains; FI, Falkland Islands; MBL, Marie Byrd Land; SG, South Georgia; TAM, Transantarctic Mountains; TI, Thurston Island.



similar to those of the Ferrar province, whereas those that erupted after sea-floor spreading was established have characteristics typical of normal ocean floor basalts (N-MORB)⁵³. An extensional basin also formed along the southeast margin of the Antarctic Peninsula during a period of Early Jurassic bimodal magmatism behind the active continental arc system⁵⁴ (Fig. 5).

Active margin of Gondwanaland. The formation of the Gondwanaland mafic and felsic provinces and extensional basins was synchronous with active-margin tectonics and development of a Pacific-margin magmatic suite^{29,46} (Fig. 5). During the Jurassic, subduction-related magmatism migrated from the eastern towards the western margin of the Antarctic Peninsula, bringing the magmatism closer to the trench and reflecting a possible change from shallow to steeply dipping subduction²⁹. The margin was clearly extensional at that time, grabens formed in the fore-arc region in advance of the westward-migrating magmatism, the arc was deformed by extensional shear zones and basins formed in the back-arc region of southern South America and Antarctica^{52,54}. These may have been contiguous with basins now preserved in the Weddell Sea embayment area and a failed rift⁵⁵, indicating a broad extensional province (Fig. 5). Structural analysis of dyke trends and associated normal faults along the Transantarctic Mountains have shown that the extension direction was normal to the proto-Pacific margin⁵⁶ and may have been controlled by changes in plate boundary forces along the active margin during subduction.

Plume or plate-boundary forces? The magmatic and tectonic record along the proto-Pacific margin of Gondwanaland seems therefore to record important changes in subduction zone forces during the initial stages of the disintegration of Gondwanaland²⁹. However, their role, together with that of a mantle plume or plumes (possibly Bouvet and Shona hotspots⁵¹) postulated to lie beneath the Karoo province before breakup, are uncertain and have been assigned different priorities by different workers^{29,51,57}. The absence of subduction along the Neotethyan margin of Gondwanaland, together with the fact that the initial rift formed nearly at right angles to the active subducting margin (Fig. 1), suggest a potential active role for mantle plumes in the initial separation of East and West Gondwanaland. However, the difference in timing, of ~26 Myr, between the initial plume-related magmatism of the Karoo province (182 ± 2 Myr) and final breakup (~156 Myr based on anomaly M22 (refs 25, 26)), make this less likely, although it should be pointed out that the age gap may not actually be as large as 26 Myr, as there is sufficient room between the identified anomalies and the continental margins to suggest that sea-floor spreading may have started at ~170 Myr (ref. 2). Moreover, the large extent of the magmatic provinces suggest that the concept of Gondwanaland being underlain by a broad shallow region of higher-than-normal mantle temperatures rather than single plumes cannot be ruled out. A 'Gondwanaland thermal anomaly', as in the Anderson model^{21,58}, may have been due to the insulating effect of the supercontinent. Either plume point sources or a regional heat anomaly could have thermally weakened the lithosphere, increased magma production rates and induced local rifting, at least contributing to the eventual separation of East and West Gondwanaland.

The gravitational potential of crust that may have been over-thickened during the Permian–Triassic Cape folding event may also have been a contributory factor, and the spatial coincidence, at least on a regional scale, of the folding and Karoo plume in southern Africa may eventually have controlled the locus of breakup²⁹. The change from compression to lithospheric extension may also have been linked, in Jurassic times, to a reduction in compressive plate-boundary forces and a change from a shallow to a steeply dipping subduction zone⁴⁶. The combination of subduction pull on one side of the continent together with the local tensional effects of a heat anomaly and collapse of over-thickened lithosphere may have been sufficient to cause breakup and final separation. In this model, the mantle plume did not

provide the essential trigger, although it may have controlled the ultimate position of breakup and of major fault systems. Uplift associated with the mantle plume may also have been responsible for the formation and migration of small continental blocks, for example Falkland Islands^{51,59}, Haag Nunataks and Ellsworth Mountains⁶⁰, from their original Gondwanaland position in the vicinity of mantle-plume heads off southern Africa, to their present positions on the South American and Antarctic plates.

An alternative view that breakup of the supercontinent was preceded by prolonged meteorite impact cratering that extensively fractured the lithosphere, initiated mantle plumes and facilitated final continental breakup^{61,62} is not generally accepted. The hypothesis is based on a reinterpretation of what most people consider to be glacial tillites formed during a Permian–Carboniferous glaciation of Gondwanaland, as the products of meteorite impacts. However, to my knowledge, a field evaluation of the critical criteria for differentiating between deposits of glacial and meteorite impact origin has not been made, and this must be considered a prerequisite for further consideration of this idea.

South America–Africa rifting

Two large-scale mantle plumes, the present-day positions of which are close to the oceanic islands of Tristan da Cunha and St Helena (Figs 1 and 4) are closely associated with the opening of the South Atlantic^{63,64}, although the cause and effect are still matters of debate. In the southern part, the Tristan da Cunha hotspot is linked by the Walvis Ridge and Rio Grande Rise to the large continental flood-basalt province of the Parana in South America and the Etendeka in western Africa⁶³ (Fig. 4). The Parana, one of the largest flood basalt provinces in the world, forms the cover of the long-lived Paraná basin⁶⁵. Although some data⁶⁶ suggested that the entire province was erupted within 1 Myr (~132 Myr), other data indicated an eruption history extending over 10 Myr between 127 and 137 Myr (ref. 67). Besides this uncertainty, we have a poor understanding of the complexities of the rifting history in the South Atlantic, where sea-floor spreading began before 133 Myr (based on the firm identity of anomaly M10 (refs 68, 69)) close to the time of plume magmatism. The spreading ridge propagated north with sea-floor spreading in the vicinity of the Tristan plume at ~124 Myr (M2), which is younger than the main phase of plume magmatism.

In the equatorial region, the St Helena plume did not generate the same volume of magmatism as the Tristan plume⁶⁴. Nevertheless, the hotspot is linked by a seamount chain to a broad zone of rifting and magmatism in western and central Africa that was initiated 30–40 Myr before sea-floor spreading began off Nigeria between 107 and 112 Myr (ref. 70) (Fig. 4). This temporal relationship, between plume magmatism and the onset of sea-floor spreading in the central Atlantic region, is similar to relationships between the Karoo plume and the initial rifting of Gondwanaland and suggests that the horizontal extensional stresses arising from the mantle plume were incapable of causing breakup⁶⁴. In contrast, in the case of the Tristan plume, there is a close temporal correlation between the onset of plume magmatism and initial sea-floor spreading, although the fact that sea-floor spreading initiated to the south and propagated northwards across the plume head argues against an active-plume mechanism.

Both the Tristan and St Helena plumes must undoubtedly have influenced the response of the lithosphere to applied stresses and played a significant and important role in determining precisely where continental breakup occurred, with the continental lithosphere breaking across the heads of both plumes^{63,64}. The Tristan plume could even have determined when breakup occurred locally, as indicated by the close temporal relationships between magmatism and the onset of sea-floor spreading off the Walvis Ridge. The plume could also have provided sufficient

extra force to drive what could have been a weak extensional system, but there is no real evidence for this.

Antarctica/Australia–India rifting

The separation of greater India from Australia was preceded by a long history of intra-cratonic rifting and magmatism that began in Late Triassic and Early Jurassic times along the western and northwestern Australian margin⁷¹. The first phase of sea-floor spreading in the Indian Ocean produced ocean floor from magnetic anomaly M25 time (~161 Myr) with separation of the Argo block from northwest Australia, and from anomaly M10–11 (~133 Myr) time in the Cuvier and Perth basins along the western margin of Australia^{72,73}. The exact age of the onset of sea-floor spreading between India and Antarctica cannot be determined more precisely than 118–128 Myr owing to the paucity of identifiable Mesozoic magnetic anomalies².

The long history of rifting was accompanied by extensive magmatism in eastern India, Kerguelen Plateau and western Australia (Fig. 6). The Rajmahal basalts (115–117 Myr) are the best known of the Indian province⁷⁴. They rest on the fill of a Permian to Jurassic extensional basin and form the leading edge of a series of seaward-dipping flows largely buried beneath a sedimentary cover in the Bengal basin⁷⁵. They were virtually contemporaneous with extrusion of the Kerguelen Plateau basalts (115–118 Myr), which, although having a continental lithosphere signature⁷⁶, essentially comprise an oceanic plateau that formed after continental separation, filling the space between India and Antarctica. The age of the Bunbury basalts in southwest Australia is constrained by a new Ar–Ar age of 115 ± 2 Myr at the same time as the extrusion of the Rajmahal basalts⁷⁷. They are also part of a laterally extensive volcanic margin of western Australia that includes the Wallaby and Naturaliste submarine plateaus^{14,71,77} (Fig. 4).

Most reconstructions of the northeast Indian Ocean are based traditionally on the assumption that the Rajmahal basalt, and the Ninetyeast Ridge (Fig. 4) were formed by the Kerguelen hotspot¹⁷, a correlation consistent with geochemical data. However, more recent reconstructions suggest that the Rajmahal basalts, the 85° E ridge and the Ajanasy Nikitin seamounts are the trace of the hotspot that now lies beneath Iles Crozet²⁷. This places the Kerguelen hotspot close to the triple junction between India, Australia and Antarctica. This correlation was disputed by Müller *et al.*²⁸, who suggested that the 85° E ridge and seamounts were formed by a hotspot now located beneath the East Conrad Rise (Fig. 4). Irrespective of the precise correlation with today's hotspots, the timing of this volcanism is too young for a plume to have been involved in the initial breakup of this margin, which occurred at ~161 Myr along the northwest margin and ~130 Myr along the western margin of Australia, unless, as suggested by Kent⁷⁵, a very large plume head existed beneath the area for up to 150 Myr before breakup. This hypothesis was based on a prolonged history of alkali magmatism, gentle doming of the crust and incipient rifting. Hopper *et al.*⁷⁸ did not propose a plume model for the western Australian margin because of its broad extent and the lack of evidence for hotspot activity, but instead favoured a model where rapid extension can induce short-lived convection in the mantle and a pulse of unusually large magmatism⁷⁹. It would therefore seem more likely that extension and continental breakup proceeded for other reasons, with pre- and syn-breakup basalts being produced by rifting, as in non-plume models, and the eventual unroofing of a plume producing the post-breakup basalt provinces. The plate-tectonic regime in the Neotethyan ocean basin off the northern margin of Australia may have been an important controlling factor here. Neotethyan ocean floor was being subducted northward beneath Eurasia at that time³¹. The approach and eventual collision of the spreading ridge with Eurasia, and the continued subduction of ocean floor beneath Eurasia, would have changed the stress regime within Gondwanaland from compressional (due to ridge push) to extensional (due to slap pull)

and could have been the dominant factor in the separation of India from Antarctica. The position of rifting could have been controlled by plumes or hot regions beneath Gondwanaland, with their eventual unroofing by plate tectonic forces producing the basalt provinces.

Australia–Antarctica rifting

In mid-Cretaceous times (~100 Myr), the southeastern Indian Ridge propagated eastwards, with initiation of sea-floor spreading (at 95 ± 5 Myr) in a region of highly extended crust between Australia and Antarctica⁸⁰. In contrast to the earlier breakup history of Gondwanaland, the separation of Australia from Antarctica was not linked to extensive volcanism. The passive margins are classified as non-volcanic and represent a classic example of continental separation, following a slow period of rifting without the influence of a mantle plume. They may, as has been suggested for the separation of India from Antarctica, have been linked to a change in the plate-tectonic regime in the Neotethyan ocean basin to the north. The long-term effect of subduction of Neotethyan ocean floor northwards beneath Eurasia³¹ may have been to pull Australia away from Antarctica.

Australia–Antarctica–New Zealand rifting

Compared with Australia–Antarctica rifting, the separation of New Zealand from both Australia and from the Marie Byrd Land sector of Antarctica (Fig. 4), was more complicated and was linked to extensive volcanism. Lanyon *et al.*⁸¹ speculated that the initial opening of the Tasman Sea was synchronous with inception of the Balleny plume in Late Cretaceous times. They linked the present-day hotspot beneath the Balleny Islands, via a series of seamount chains, to an original position on the western margin of the Lord Howe Rise. Intensification of plume activity was related to the formation of the Tertiary alkaline volcanism in Tasmania coincident with the marked increase in spreading rates between Australia and Antarctica at 45 Myr.

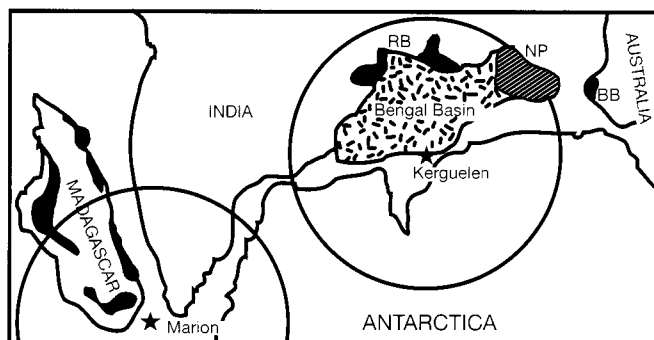
The time of separation of New Zealand from the Marie Byrd Land sector of Antarctica (Fig. 5) is constrained by the oldest identified oceanic magnetic anomaly (C34, ~84 Myr)⁸² and was preceded by a rapid transition from arc- to rift-related anorogenic magmatism in Marie Byrd Land in mid-Cretaceous times (at ~100 Myr)⁸³. Although the rift-related magmatic rocks do not have an obvious plume signature, Weaver *et al.*⁸³ speculated that the mantle plume known to exist beneath the Cenozoic alkaline basalt field in Marie Byrd Land⁸⁴ may have existed in Cretaceous times and controlled the position of breakup. A Cretaceous plume would also account for the contrast between the uplifted thermally buoyant Marie Byrd Land margin with its voluminous anorogenic granitoids⁸³ and unroofed metamorphic complexes⁸⁵, and the conjugate New Zealand margin with its Late Cretaceous sedimentary basins containing up to 4.6 km of sediment.

The mid-Cretaceous was also a time of change in the plate-tectonic regime along the New Zealand margin with a consequent change in plate-boundary forces. Subduction ceased at ~105 Myr, after collision of the Pacific–Phoenix spreading ridge with the trench⁸⁶ (Fig. 1d). Continued divergent motion between the Pacific and Antarctic plates resulted in the development of a new extensional plate boundary, the Antarctic–Pacific ridge between New Zealand and Antarctica. The postulated mantle plume could have thermally weakened the crust, and influenced the ultimate position of breakup⁸³.

Africa–India rifting

In Late Cretaceous and Tertiary times, India migrated rapidly northwards (15 cm yr^{-1}) away from Africa with sea-floor spreading taking place not, as one might have expected, in the existing Mozambique basin, but within the Mascarene basin between 63 and 80 Myr (C28–C33)¹⁴. This resulted in the separation of Madagascar from India (Figs 4 and 6). Plate reconstructions for the Late Cretaceous place Madagascar over the Marion

FIG. 6 A composite diagram illustrating the location of the Rajmahal basalts (RB), Bunbury basalts (BB), Naturaliste Plateau (NP) and the possible extent of basalt within the Bengal basin (stippled) together with the location of the Kerguelen plume (2,000 km in diameter), ~118 Myr (refs 28, 75). The Kerguelen Plateau formed as soon as India separated from Antarctica. The diagram also shows the position of the Marion plume head (also 2,000 km in diameter) and the basalt province in Madagascar before separation of Madagascar from India, ~88 Myr (ref. 87).



plume, a position confirmed by the presence of an extensive flood-basalt province (Fig. 6) that has a mean age of 88 Myr (Fig. 6) and that was emplaced over no more than 6 Myr (ref. 87). After C28, spreading ceased in the Mascarene basin but was replaced by a spreading system that split the Seychelles away from the Indian mainland (Fig. 7) and created the eastern part of the Somali basin^{14,88}. This rifting event was closely preceded by and overlapped with formation of the Deccan continental flood basalt province in western India at 69–65 Myr (ref. 89) (Fig. 7). The basalts mark the former position of the Réunion plume, to which it is linked by the Chagos–Laccadive and Mascarene ridges (Fig. 4). Although relationships between magmatism, rifting and sea-floor spreading have been used in support of both active and passive models of rifting^{14,89}, the close temporal and spatial coincidence of rifting with basalt provinces and the separation of both Madagascar and the Seychelles from India suggest that at least the position of breakup was controlled by the Marion and Réunion plumes. In fact, in these cases, it is difficult to see how the relatively small continental blocks of Madagascar and the Seychelles could have separated from India without the active participation of the mantle plumes.

Breakup models

The breakup and dispersal of the Gondwanaland supercontinent illustrates varied temporal and spatial relationships that exist between continental rifting and magmatism, and these have important implications for breakup models. At one extreme, the separation of Australia from Antarctica proceeded without magmatism and cannot be linked to the activity of a mantle plume. At the other extreme, the temporal coincidence between plume-related magmatism and the separation of both Madagascar and the Seychelles from India suggest that, in these cases, plumes may have played an important role in continental separation. Although at this stage in our understanding it is difficult

to separate the true cause and effect, these examples most closely resemble active-plume models, and it is difficult to imagine how Madagascar and the Seychelles both separated from India without the influence of a mantle plume. In other situations, for example, the initial stage of Gondwanaland breakup, South America–Africa rifting and India–Australia rifting, the temporal relationships between plume magmatism and sea-floor spreading are such that plumes are less likely to have caused breakup but they may have had an influence in controlling its position.

A consideration of the plate-tectonic regime also suggests that the initial stages of breakup and the subsequent separation of New Zealand from Antarctica were coincident with, and may have been linked genetically to, changes in plate-boundary forces and in the plate-tectonic regime during breakup.

The debate about a plume or non-plume origin for continental flood-basalt provinces, and a plume or non-plume view of mantle dynamics, is by no means over. The profusion of flood-basalt provinces in the Indian Ocean and the South Atlantic region, and the presence of magmatic province spatially unrelated to known mantle hotspots, for example Ferrar province, suggest that the Gondwanaland continent could have been underlain by a large shallow thermal anomaly^{21,58} (Gondwana thermal anomaly) rather than by distinct hotspots fed by deep mantle plumes. One resolution of this apparent conflict is that a regional heat anomaly could be fed by deep-sourced mantle plumes incubating beneath a supercontinent, with the heat unable to escape because of the continent's insulating effect. If this was so, the combination of changes in plate-boundary forces and uplift above a regional heat anomaly could have been sufficient to cause breakup. Alternatively, non-plume models for production of some large magmatic provinces could be correct⁷⁹. There has also been a tendency in the past to consider plumes and subduction plate boundaries separately. However, the possibility exists that plumes and subducting plate boundaries could occasionally

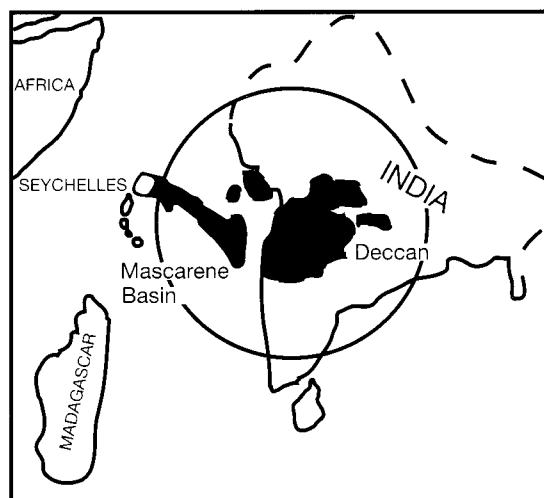


FIG. 7 The known extent of the Deccan basalt province and contemporaneous offshore basalts, and the location of the Réunion plume head (2,000 km in diameter) before separation of Seychelles from India, ~65 Myr (ref. 14).

have interacted, producing a new set of breakup parameters. The initial separation of East and West Gondwanaland in the proto-Weddell Sea region may be a case in point⁹⁰, and this needs to be tested.

The many debates concerning continental breakup can be brought forward only by improved imaging of mantle flow and mantle temperature regimes, leading to a better understanding of mantle dynamics. The questions of the size, duration and distribution of thermal anomalies in the mantle are important and need to be answered. Further constraints on these models can also come with more precise dating of the magmatic pro-

vinces and the onset of sea-floor spreading, by more clearly defining plume signatures in the large magmatic provinces and by investigating the thermal regime at their time of emplacement. Of most importance, however, is to achieve a better understanding of the whole plate-tectonic regime at the time of breakup. Only then will we be in a better position to assess the relative contributions of deep mantle processes and changes in the plate-tectonic regime to the breakup process. □

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A base pair between tRNA and 23S rRNA in the peptidyl transferase centre of the ribosome

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Interaction of the conserved CCA terminus of tRNA with rRNA in the peptidyl transferase P site has been studied by *in vitro* genetics. A Watson–Crick G–C pair between G2252 in a conserved hairpin loop of 23S rRNA and C74 at the acceptor end of tRNA is required for proper functional interaction of the CCA end of tRNA with the ribosomal P site. These findings establish a direct role for 23S rRNA in protein synthesis.

PROTEIN synthesis is mediated by transfer RNA, the anticodon sequences of which recognize complementary codon sequences in messenger RNA in the structural context of the ribosome. In addition to this specific tRNA–mRNA interaction, tRNA also interacts with the ribosome itself^{1,3}. Evidence from crosslinking and chemical footprinting experiments has suggested that tRNA–rRNA interactions could be involved in tRNA binding by the ribosome^{4,12}. Recent modification-interference experiments have identified bases in 16S rRNA that are essential for binding of the anticodon stem-loop end of tRNA to the P site of the small ribosomal subunit¹³. Here we investigate the interaction of the acceptor end of tRNA with 23S rRNA in the large subunit.

All known tRNAs contain the sequence CCA at their 3' mature ends. Early studies showed that this universally conserved sequence is essential for a proper functional interaction between tRNA and the peptidyl transferase P site. For example, it has been shown¹⁴ using aminoacyl-oligonucleotide analogues of peptidyl-tRNA that the 3'-aminoacylated CCA moiety is necessary and sufficient for productive binding to the large-subunit P site¹⁴. Consistent with this, chemical protection studies have shown that the two conserved cytosines are protected from attack by dimethyl sulphate when tRNA is bound to the ribosomal P site^{15,16}.

Sequences in 23S rRNA show similarly strong conservation, and several lines of experimental evidence implicate specific sites in 23S rRNA in interaction with the CCA sequence of P-site tRNA. Among the bases in 23S rRNA that are protected from chemical probes by P-site tRNA and/or CCA-containing oligonucleotide analogues of P-site tRNA are the universally conserved nucleotides G2252 and G2253 (refs 9, 10). Stepwise deletion experiments have shown that deletion of C75, but not A76, of tRNA specifically results in loss of protection of these two bases. This result suggested possible base–base interaction between the conserved cytosines of tRNA with the conserved guanines in 23S rRNA⁹.

Mutations at G2252 and G2253 have been shown to have dominant lethal and recessive slow-growth phenotypes, respectively, indicating an essential role for these bases that is consistent with their possible involvement in tRNA–ribosome interaction¹⁷ (R.R.S., unpublished data). Further support for this comes from the finding that ribosomes containing mutations in these two bases are impaired in the peptidyl transferase reaction¹⁷ (see below).

In the absence of a simple *in vivo* approach to test these potential base–base interactions, we devised two different strategies to analyse the binding and catalytic properties of mutant ribosomes

interacting with a set of mutant tRNA substrates. First, we exploited the characteristic footprint that is observed upon binding of the CCA end of tRNA to the P site of the large ribosomal subunit^{9,10}. Hydrogen-bonded interactions between RNA bases protect the interacting atomic positions from attack by chemical probes such as kethoxal (at the N1 and N2 positions of guanine) and 1-cyclohexyl-3 (2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate (CMCT) (at the N3 position of uridine), providing a means to directly monitor interactions involving specific bases in rRNA.

Ribosomes carrying mutations at positions G2252 or G2253 of 23S rRNA were tested for their ability to interact with tRNA or aminoacyl-CCA oligonucleotide analogues carrying either wild-type or mutant sequences. Interaction with the large subunit P site was monitored by tRNA-dependent protection of G2252 or G2253 from kethoxal modification, or by protection of U2585 from the carbodiimide reagent CMCT¹⁸. The extent of chemical modification or protection was assayed by primer extension with reverse transcriptase, which stops or pauses when it encounters a modified base. Binding of tRNA to mutant ribosomes could be distinguished from binding to wild-type ribosomes, which are unavoidably present in comparable amounts, by allele-specific primer extension^{19,20}; silent mutations are introduced into a cloned copy of 23S rRNA, allowing specific DNA oligonucleotide primers to be used to distinguish between the modification patterns of 23S rRNA in mutant versus wild-type ribosomes (Fig. 1).

Second, we developed an *in vitro* approach for measuring directly the peptidyl transferase activity of ribosomes containing mutations in 23S rRNA. This has not been possible until now because pure mutant rRNA cannot be generated *in vivo*, and *in vitro* transcripts of *Escherichia coli* 23S rRNA are not functional in *in vitro* reconstitution reactions. A 3' RNase H fragment of natural 23S rRNA is combined with an *in vitro* transcript of the corresponding 5' fragment of 23S rRNA in a chimaeric reconstitution reaction with large-subunit proteins and 5S rRNA to yield catalytically active 50S subunits. By using this assay we have examined the ability of mutant oligonucleotide substrates to suppress the peptidyl transferase deficiencies of ribosomes containing mutations in 23S rRNA at position 2252.

Mutations at G2252

Mutations were introduced at positions 2252 and 2253 of *E. coli* 23S rRNA genes as part of an *rrnB* operon-containing plasmid construct under expression from either the native P₁P₂ rRNA promoter, or from an inducible lambda P_L promoter. In agreement with previous results²¹, we were unable to isolate transformants containing mutations at position 2252 under expression from the strong P₁P₂ promoter. Expression of 23S

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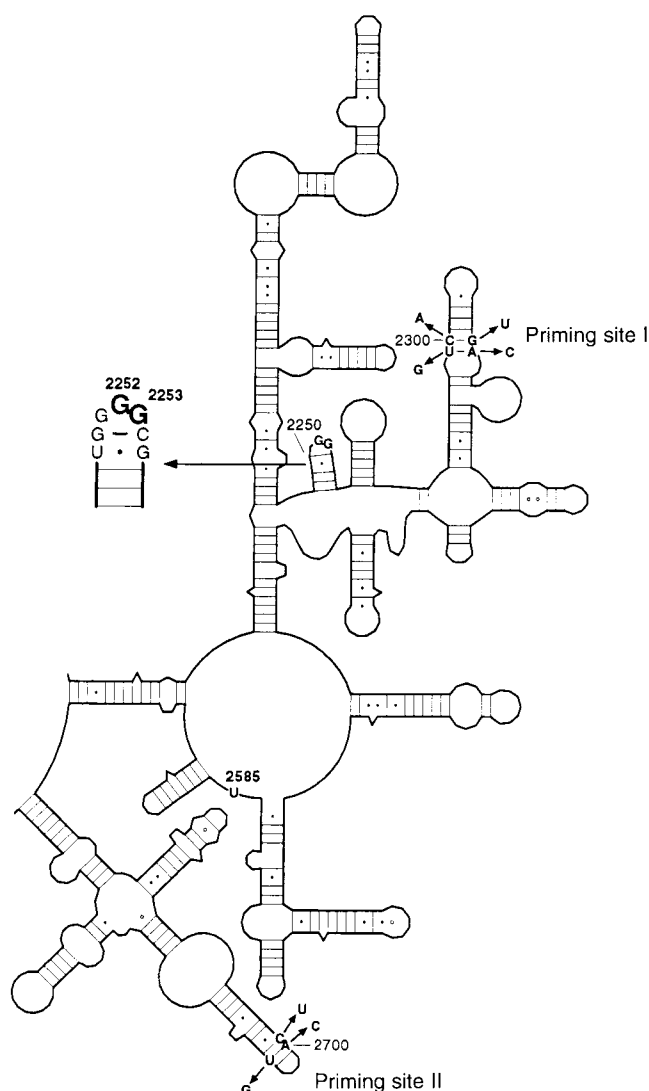


FIG. 1 Schematic drawing of the secondary structure of domains V and VI of *E. coli* 23S rRNA³¹, showing the locations of nucleotides G2252 and G2253. Also shown are silent mutations introduced at the two allele-specific priming sites^{19,20} I and II that were used to monitor tRNA or oligonucleotide interactions at positions G2252–2253 or U2585, respectively.

METHODS. An *EcoRI*–*KpnI* fragment from pSTLKpn (a derivative of pSTL102 (ref. 22) in which a single *KpnI* site was engineered at the end of the 23S rRNA coding region) was ligated into Bluescript (Stratagene) to generate plasmid pBS23SRS. Oligonucleotide-directed mutations were constructed in pBS23SRS³² modified for use with Bluescript³³. Mutations were identified by dideoxy sequencing³⁴. The following primers were used in these studies; when multiple sites were being mutagenized simultaneously, the respective primers were added together at the hybridization step: G2252A, 5′-GGGTAGTTTGACTGGA-GCGGTCTCC-3′; G2252C, 5′-GGGTAGTTTGACTGGCGCGGTCTCC-3′; G2252U, 5′-GGGTAGTTTGACTGGUGCGGTCTCC-3′; G2253A, 5′-GGGTAGTTTGACTGGGACGCTCTCC-3′; G2253U, 5′-GGGTAGTTTGACTGGGUCGGTCTCC-3′; primer site I, 5′-GGCUAAGACUGGGACAUCAGUCGG-UUAGUC-3′; primer site II, 5′-CGGGUUCUGCCAAGGCGACUGCCCG-3′. Plasmid construction; mutant derivatives of pBS23SRS were digested with *Asp* 718 (Boehringer-Mannheim) and ligated to *Bam*HI linkers (New England Biolabs). The resulting linear plasmid was digested with *Bam*HI and *Sph*I and introduced into plasmid pLK45 containing the *rrnB* operon under control of phage lambda P_L promoter³⁵. The resulting plasmids were transformed into *E. coli* strain DH1 containing plasmid pcl857 which encodes a thermolabile allele of the lambda repressor. Resulting pLK plasmids containing the respective mutations and custom priming sites were selected on plates containing both ampicillin (40 mg l⁻¹) and kanamycin (30 mg l⁻¹). Plasmids containing only priming sites I and II were also generated, and the growth rates of *E. coli* DH1 cells containing these or wild-type pLK45 plasmids were measured and found to be similar.

rRNA containing mutations at 2252 from the less efficient P_L promoter caused dramatic inhibition of growth. Because the transformed cells contain comparable amounts of wild-type and mutant ribosomes under these conditions, the negative effect of the 2252 mutations is dominant. Mutation of position 2253 produced no such dominant effect, but a recessive slow-growth phenotype was apparent when cells were grown on erythromycin-containing media, which restricts protein synthesis to ribosomes containing plasmid-encoded 23S rRNA, carrying an erythromycin-resistance marker³².

The tRNA-dependent protection of U2585 from CMCT modification requires an intact CCA end, and specifically the presence of the 3′-terminal adenosine^{9,10}. Mutation of G2252 to A affects U2585, decreasing its reactivity towards CMCT (Fig. 2a, b). In addition, the ability of wild-type tRNA^{Phe} to protect U2585 is significantly reduced (Fig. 2a, b). The ability of a point mutation at position 2252 to affect the reactivity of a base more than 300 nucleotides away provides compelling evidence that the 2250 and 2580 regions of 23S rRNA are somehow structurally linked^{9,10,23,24}. Moreover, it is clear that the binding affinity of wild-type tRNA^{Phe} or *N*-Acetyl-Phe-tRNA is similar for the mutant and wild-type ribosomes (Fig. 2c, d), probably because binding of tRNA^{Phe} to poly(U)-programmed ribosomes is dominated by its interactions with the 30S subunit²⁵.

Binding of CCA(AcPhe) oligonucleotides

Because ribosomes containing the G2252A mutation retain their ability to bind tRNA^{Phe}, we sought a more sensitive assay for

interaction of the CCA end of tRNA with the 50S ribosomal P site, where the 50S interaction has a greater relative contribution. Early studies¹⁴ had shown that N-blocked aminoacyl oligonucleotide fragments containing the 3′ CCA sequence, such as CAACCA(f-Met) or CACCA(Ac-Phe), are substrates for peptidyl transferase under the conditions of the 'fragment reaction'. It was also shown that these oligonucleotide fragments can be bound stably to 50S ribosomal subunits in the presence of 33% methanol and sparsomycin²⁶. Chemical footprinting studies have shown that fragment binding confers virtually the same pattern of protection of bases in 23S rRNA as that observed for binding intact peptidyl-tRNA¹⁰. The requirements for peptidyl transferase activity and binding were shown to be similar; the 5′ ends of the oligonucleotides could be deleted without effect, but deletion of C74, to give CA(f-Met), for example, abolished both activity and binding. Thus the minimal P-site substrate has an intact CCA sequence and a blocked aminoacyl moiety.

Using the protection of U2585 from CMCT as an assay, we tested the binding of mutant versions of the oligonucleotide fragment CACCA(Ac-Phe). Mutation of either of the two cytosines, corresponding to C74 or C75 of tRNA, caused significant loss of binding to wild-type ribosomes (Fig. 3, WT) in keeping with their universal conservation in all known mature tRNA sequences. Conversely, mutation of G2252 to U resulted in loss of binding of the wild-type oligonucleotide fragment (Fig. 3, G2252U). Binding to ribosomes containing the G2252U mutation was recovered using the mutant oligonucleotide fragment CAACA(Ac-Phe), but not with several other oligonucleotides

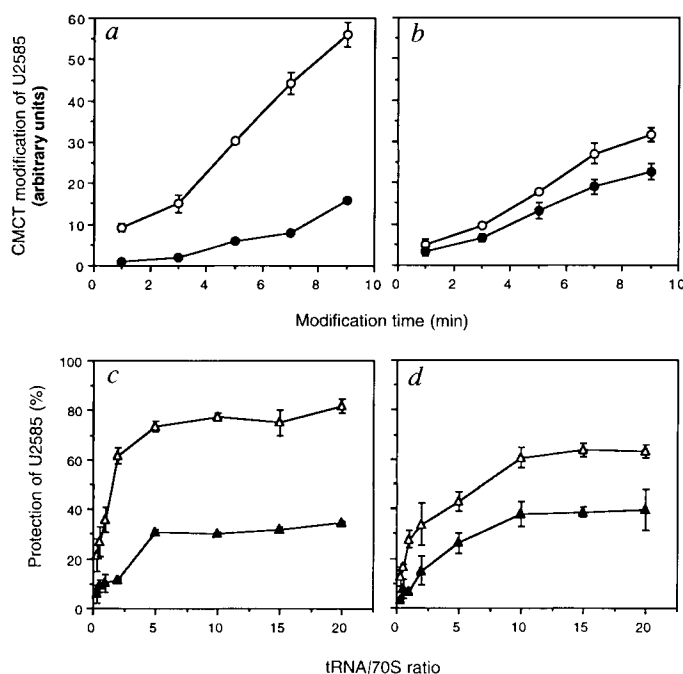


FIG. 2 a, b, Kinetics of modification of U2585 in 23S rRNA by CMCT in wild-type (a) and G2252A mutant (b) ribosomes. Open circles, vacant ribosomes; filled circles, ribosomes with tRNA^{Phe} bound in the presence of poly(U). c, d, Titration of wild-type (open triangles) and G2252A (filled triangles) ribosomes with deacylated (c) or N-Acetyl-Phe-tRNA (d), assayed by protection of U2585 from modification by CMCT.

METHODS. a, b, 60 pmol *E. coli* 70S tight-couple ribosomes were incubated with 6 A₂₆₀ units of poly(U) in 150 μ l 70 mM K-borate (pH 8.0), 20 mM MgCl₂, 100 mM NH₄Cl and 6 mM DTT (KMND) buffer for 10 min at 37 °C, followed by addition of 300 pmol deacylated tRNA^{Phe} in 150 μ l of the same buffer and incubation for an additional 30 min. CMCT (300 μ l) in the same buffer was added, and 50- μ l portions were removed at the indicated times and transferred to 50 μ l 0.3 M NaOAc, pH 5.0. Isolation of the modified RNA and allele-specific primer extension^{19,20} from priming site II (Fig. 1) were performed as described¹⁸, and the extent of modification of U2585 was estimated using a Molecular Dynamics Phosphorimager. c, d, 5 pmol 70S ribosomes were incubated with 0.4 A₂₆₀ units of poly(U) in 25 μ l KMND at 37 °C for 10 min followed by addition of increasing amounts of deacylated tRNA^{Phe} or N-acetyl-[¹⁴C]-Phe-tRNA in 25 μ l of the same buffer and incubation for an additional 30 min. tRNA binding was assayed by protection from CMCT modification, monitored by primer extension as described above. Aminoacylation and acetylation of tRNA^{Phe} were performed as described previously⁹. N-acetyl-Phe-tRNA was purified by BD-cellulose column chromatography as described³⁶.

containing mutations at either the C74 or C75 positions (Fig. 3, G2252U). Wild-type oligonucleotide also failed to bind to ribosomes carrying a G2252A mutation; in this case, loss of binding was suppressed by mutation of the oligonucleotide sequence to CAUCA(Ac.Phe), but not by other mutant fragments (Fig. 3, G2252A).

Thus oligonucleotide binding to the 50S P site was observed only when the relationship between position 2252 of 23S rRNA and position 74 of the tRNA fragment was G-C, A-U or U-A (the C-G pairing possibility could not be tested in this assay because the compensatory oligonucleotide introduces a G, which is cleaved by RNase T₁ during preparation of the oligonucleotide); other combinations gave no binding or only weak binding (Fig. 3). Mutations at position 75 of the tRNA fragment failed to rescue binding to ribosomes bearing mutations at position 2253 (Fig. 3, G2253U and G2253A). These results provide evidence that binding of the CCA oligonucleotide to the peptidyl transferase P site requires a Watson-Crick interaction between C74 of tRNA and G2252 of 23S rRNA.

In contrast, mutation of G2253 had little or no effect on binding of the wild-type oligonucleotide fragment, nor was any

additional suppression observed with any of the mutant oligonucleotide fragments on ribosomes containing either the G2253U or G2253A mutations (Fig. 3). Thus, although both C75 and G2253 are also universally conserved bases, and both are protected from chemical probes when tRNA is bound to the ribosomal P site^{9,10,15,16}, there is no obvious requirement for base pairing between them for binding of the CCA oligonucleotide fragment.

Binding of intact tRNA

We also tested the ability of wild-type and mutant versions of full-length tRNA to interact properly with the 50S P site of mutant ribosomes, to see whether the Watson-Crick relationship observed for the CCA oligonucleotide also holds for intact tRNA, and to exclude the possibility that our results were influenced by the presence of methanol and sparsomycin, which are required for oligonucleotide binding. Here we could not rely on tRNA binding itself because, as discussed above, binding of tRNA under these conditions is dominated by interactions with the 30S subunit. Instead, we used protection of G2253 (or G2252) from kethoxal as an assay for the localized interaction

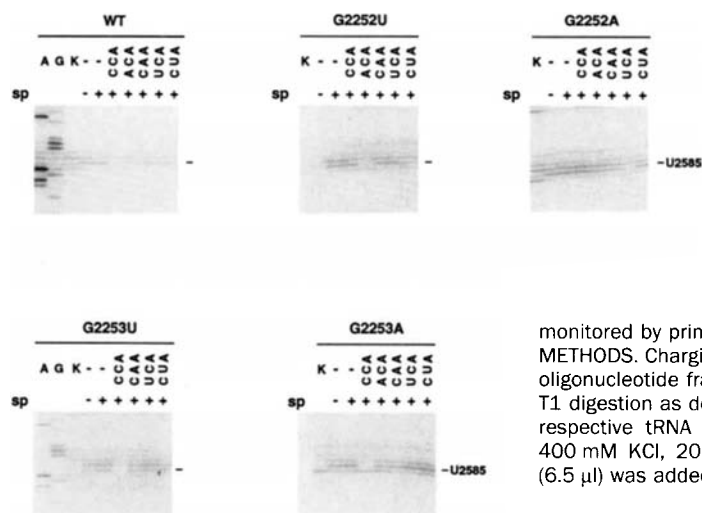


FIG. 3 Binding of mutant and wild-type aminoacyl-oligonucleotides to the P site of 50S ribosomal subunits containing mutant or wild-type 23S rRNA, assayed by protection of U2585 from attack by CMCT. WT indicates wild-type 23S rRNA, and G2252U, G2252A, G2253U and G2253A are the corresponding mutant 23S rRNAs. A and G indicate sequencing lanes, K is unmodified 23S rRNA, sp indicates the presence (+) or absence (-) of sparsomycin. CCA indicates the wild-type tRNA oligonucleotide fragment CACCA(Ac-Phe), and ACA, CAA, UCA and CUA the corresponding mutant versions of the oligonucleotide. Chemical reactivity of U2585 was monitored by primer extension from priming site II as described in Fig. 2.

METHODS. Charging and N-acetylation were performed as described previously^{9,36}. tRNA oligonucleotide fragments containing 2',3'-linked N-acetyl-Phe were prepared by RNase T₁ digestion as described¹⁰. Tight-couple 70S ribosomes (2.5 pmol) and 25 pmol of the respective tRNA fragment were incubated in 12.5 μ l 80 mM K-cacodylate (pH 7.2), 400 mM KCl, 20 mM MgCl₂, and 0.1 mM sparsomycin on ice for 15 min. Methanol (6.5 μ l) was added to initiate binding, and incubation was continued for 2 h.

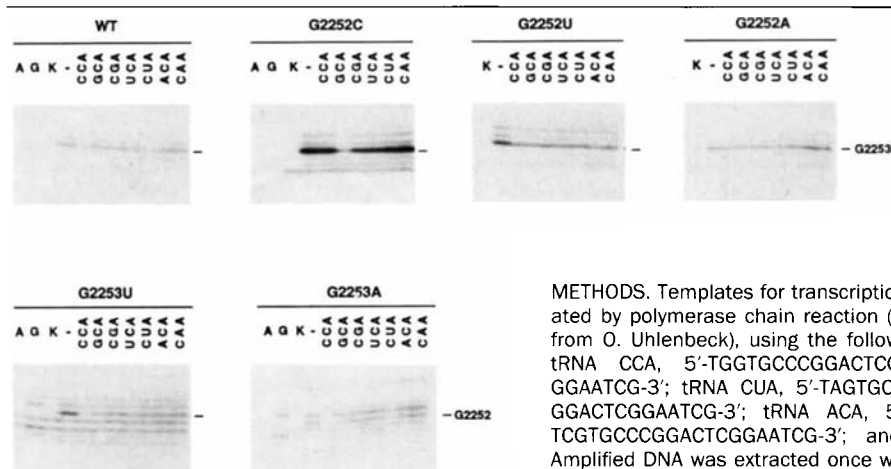


FIG. 4 Binding of mutant and wild-type tRNA^{Phe} to the P site of 50S ribosomal subunits, assayed by protection of G2253 (or 2252) from attack by kethoxal. Designations are as in Fig. 3, except that CCA refers to wild-type tRNA, and GCA, CGA, UCA, CUA, ACA and CAA are tRNAs bearing mutations in the 3' terminal CCA sequence. Kethoxal reactivity of G2253 (or 2252) was monitored by primer extension from priming site I (Figs 1 and 2).

METHODS. Templates for transcription of mutant (CCA) and wild-type tRNAs were generated by polymerase chain reaction (PCR) amplification of CF23 plasmid (ref. 37; a gift from O. Uhlenbeck), using the following primers: T7 top, 5'-TAATACGACTCACTATAG-3'; tRNA CCA, 5'-TGGTGCCCGGACTCGGAATCG-3'; tRNA UCA, 5'-TGATGCCCGGACTCGGAATCG-3'; tRNA CUA, 5'-TAGTGCCCGGACTCGGAATCG-3'; tRNA CAA, 5'-TTGTGCCCGGACTCGGAATCG-3'; tRNA ACA, 5'-TGTGTCGCGGACTCGGAATCG-3'; tRNA GCA, 5'-TCGTGCCCGGACTCGGAATCG-3'; and tRNA CGA, 5'-TCGTGCCCGGACTCGGAATCG-3'. Amplified DNA was extracted once with phenol and once with chloroform, concentrated on a Microcon ultrafilter (Amicon), and transcribed *in vitro* with T7 polymerase³⁸. tRNA

transcripts were purified on a 10% denaturing polyacrylamide gel and eluted overnight in 0.3 M NaOAc at 4 °C. Eluted tRNAs were recovered by ethanol precipitation and resuspended in water. Before use, tRNAs were renatured by heating at 95 °C for 2 min followed by slow cooling to room temperature. MgCl₂ was added to a final concentration of 10 mM. *E. coli* 70S ribosomes (10 pmol) plus 1 A₂₆₀ unit of polyU were incubated in 25 µl of 80 mM K-cacodylate (pH 7.2), 20 mM MgCl₂, 100 mM NH₄Cl and 6 mM DTT at 37 °C for 10 min. The respective tRNA (150 pmol) in 25 µl of the same buffer was added and the incubation continued for an additional 30 min. Kethoxal modification and primer extension from priming site I were performed as described in Fig. 2.

of the CCA end of tRNA with G2252 (or G2253) in the 50S P site. This strategy is based on our earlier observation that deletion of C75 results in loss of protection of both G2252 and G2253 (ref. 9).

Wild-type ribosomes show strong protection of G2253 by wild-type tRNA and by mutant tRNA containing CUA at its 3' end (Fig. 4, WT), as well as modest protection by several other mutant tRNAs. We interpret the partial protection of wild-type 23S rRNA by mutant tRNAs to be a consequence of the fact that all tRNAs are tethered to the ribosome by means of their interactions with the mRNA-programmed 30S subunit, which probably positions their 3' ends in close proximity to the 50S P site.

The effects of a G2252C mutation, which could not be studied using the oligonucleotide assay described above (see above), could be tested using intact tRNA. In this case, only the mutant tRNA containing a GCA sequence at its 3' end conferred protection (Fig. 4, G2252C).

In G2252U mutant ribosomes, protection of G2253 by wild-type tRNA drops to the nonspecific background level, whereas decreased reactivity is detected only with the ACA mutant tRNA (Fig. 4, G2252U). Similarly, with G2252A mutant ribosomes, only UCA tRNA gives protection that is significantly stronger than background (Fig. 4, G2252A). In this assay the chemical protections tend to be less striking, owing to the diminished

reactivity of G2252 and G2253 in the mutant ribosomes and higher background binding caused by interaction of tRNA with the 30S subunit. Nevertheless, the same Watson-Crick relationship between C74 and G2252 is apparent. Thus all four possible Watson-Crick combinations between C74 and G2252 support interaction of tRNA with the large subunit P site.

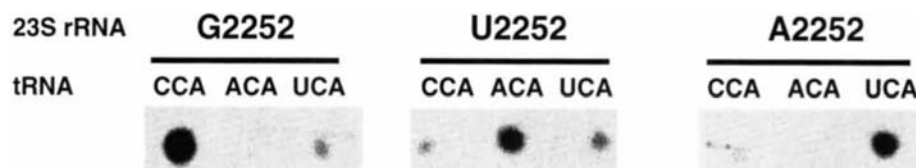
In agreement with the oligonucleotide experiments, no clear pattern of suppression is observed between mutations at position 2253 of 23S rRNA and mutations at positions C74 or C75 of tRNA. Instead, strong protection of G2252 by wild-type tRNA is observed for both the G2253U and G2253A mutant as well as for wild-type ribosomes (Fig. 4, WT, G2253U and G2253A). In addition, CUA mutant tRNA protects both wild-type and G2253A ribosomes nearly as efficiently as wild-type tRNA. Little or no significant protection above background levels is observed for any of the other mutant tRNAs, and no obvious pattern of suppression is found for any of the mutant tRNA-rRNA combinations. Thus the nature of the interaction(s) responsible for protection of C75 and G2253 must differ from that of the C74-G2252 pair.

Peptidyl transferase activity

Using a chimaeric reconstitution approach, we next addressed the importance of the Watson-Crick interaction between C74 of tRNA and G2252 of 23S rRNA for the peptidyl transferase

FIG. 5 Peptidyl transferase assay. Phosphorimager exposure of paper electrophoresis analysis of the peptidyl transferase reaction catalysed by the wild-type (G2252) and mutant (A2252 and U2252) 23S rRNA chimaeric reconstitution reactions using the wild-type (CCACCA-N-Ac-[³⁵S]-Met) and mutant (CCAACA-N-Ac-[³⁵S]-Met and CCAUCA-N-Ac-[³⁵S]-Met) aminoacylated tRNA substrates. Spots represent the product N-Ac-[³⁵S]-Met-puromycin.

METHODS. Aminoacylated fragments were prepared essentially as described¹⁰ except that elongator tRNAs were transcribed *in vitro* by T7 RNA polymerase from PCR DNA amplified from *E. coli* genomic DNA²⁹. The different tRNA fragments had the following mobilities relative to xylene cyanol FF: CCACCA-N-Ac-[³⁵S]-Met, 0.41; CCAACA-N-Ac-[³⁵S]-Met, 0.53; and CCAUCA-N-Ac-[³⁵S]-Met, 0.71. Peptidyl transferase assay was performed essentially as described³⁹ except that 20-pmol equivalents of full-length 23S rRNA (in the form of a reconstitution reaction) and 2.5 pmol of each tRNA fragment were added to a volume of 100 µl reaction. Shown in each lane is a 40-min time point. The reconstitutions



were performed essentially as described³⁰. Briefly, in a total volume of 20 µl, 0.5 A₂₆₀ units of full-length 23S rRNA (0.4 A₂₆₀ units of the 5' domain and 0.1 A₂₆₀ units of the 3' domain resulting from cleavage at 2310) and 0.02 A₂₆₀ units of 5S rRNA (Boehringer Mannheim) were incubated with 1.2 equivalents of TP50 (total protein from 50S subunits) in 20 mM Tris 7.4, 4 mM Mg(OAc)₂, 400 mM NH₄Cl, 0.2 mM EDTA, and 5 mM 2-mercaptoethanol at 44 °C for 30 min. The concentration of Mg-acetate was then raised to 20 mM and a second incubation step was performed at 50 °C for 90 min. The resulting mixture was then added directly to a peptidyl transferase reaction and the products analysed by ethyl acetate extraction followed by paper electrophoresis at pH 2.0 in formic acid (0.5 M)³⁹.

reaction catalysed by the 50S subunit. By optimization of the signal to noise ratio in the 'fragment reaction'¹⁴, we were able to detect peptide bond formation in ribosome populations having very low levels of activity. Puromycin, an aminoacyl-tRNA analogue, acts as a nucleophile to attack an N-blocked-aminoacylated fragment of tRNA in the P site, in this case the 3' terminus of elongator tRNA methionine, CCACCA-N-Ac-[³⁵S]-Met, to form as the product N-Ac-[³⁵S]-Met-puromycin. Separation of the ribosome-catalysed product from an excess of non-enzymatic hydrolysis and methanolysis products allows the detection of peptidyl transferase activities five orders of magnitude below that of native 50S subunits (R.G. and H.F.N., unpublished data).

The increased sensitivity of this assay has allowed the development of a new method of analysing the peptidyl transferase activity of mutant 23S rRNAs assembled into 50S subunits *in vitro*. *In vivo* expression of mutant 23S rRNAs results in a mixture of mutant and wild-type ribosomes, precluding direct measurement of the activity of the mutant ribosomes. Our method exploits the fact that functional large subunits of the ribosome (50S subunits) can be reassembled *in vitro* from the individual protein and RNA components in a two-step reconstitution procedure²⁷. However, attempts to assemble *in vitro* transcripts of *E. coli* 23S rRNA generated by T7 RNA polymerase have so far been unsuccessful, possibly because of missing post-transcriptional modifications (R.G. and H.F.N., unpublished data). We have circumvented these limitations by cleaving 23S rRNA into two fragments, one of which (that containing the site of interest) can be replaced by an *in vitro* transcript. The two fragments are then assembled into functional 50S particles.

RNase H cleavage was directed to positions 2295–2314 of 23S rRNA with a complementary DNA oligonucleotide²⁸, and the two resulting natural rRNA fragments were isolated on sucrose gradients. *In vitro* transcripts of the appropriate 5' region of 23S rRNA were generated by standard procedures²⁹. When the natural 3' fragment of 23S rRNA (extending from position ~2315 to 2904) is combined with either the natural 5' fragment of 23S rRNA (extending from position 1 to ~2314) or the corresponding *in vitro* transcript in a standard reconstitution mixture with 50S proteins and 5S rRNA³⁰, catalytically active particles are obtained. Overall, the catalytic activity of the particle reconstituted from fragmented 23S rRNA (both fragments native) is low, approximately 0.2% of that of natural 50S subunits. However, most of this loss of activity is not the result of fragmentation (R.G. and H.F.N., unpublished data). Importantly, no catalytic activity is detected when any of the various partial fragments are reconstituted alone.

Wild-type (G2252) and two mutant (A2252 and U2252) *in vitro* transcripts extending from nucleotide positions 1–2314 of 23S rRNA were combined in reconstitution reactions with natural partial 23S rRNA (nucleotide positions 2315–2904) to generate 50S particles. The peptidyl transferase activity of the reconstituted subunits was assayed using N-Ac-Met fragments with mutations at position C74 (CCACCA, CCAACA and CCAUCA) as P-site substrates (Fig. 5). As expected for a simple Watson–Crick interaction, the wild-type sequence (G2252) in 23S rRNA preferentially catalyses peptidyl transfer with the wild-type tRNA sequence (C74) (and next prefers the wobble base combination (U74)), the U2252 23S rRNA prefers the A74 mutant tRNA oligonucleotide and the A2252 23S rRNA prefers the U74 mutant tRNA oligonucleotide. Notably, the discrimination displayed by the wild-type chimaeric reconstitution cannot be distinguished from the discrimination displayed by a full-length wild-type natural 23S rRNA reconstitution.

Peptidyl transferase assays were performed under subsaturating conditions (for the P-site substrate) in which the relative contributions of the Michaelis constant, K_m , and the catalytic rate constant, k_{cat} , cannot be distinguished. The A site was saturated with puromycin in both the wild-type and mutant reactions (data not shown). Because active ribosomes are present at an

unknown concentration dependent on the efficiency of each reconstitution reaction, we cannot make direct comparisons between the activities of different mutant ribosomes with the same tRNA substrate. Nevertheless, our data demonstrate a clear change in substrate specificity of each mutant ribosome for the different tRNA substrates (Fig. 6a). The level of discrimination displayed by the various rRNA mutants for the different tRNA oligonucleotide substrates is substantial (at least tenfold for A2252 on UCA relative to ACA). The discrimination observed in the footprinting experiments (essentially a K_d effect) is also large, although the high background of the primer extension assay (~20%) precludes observation of differences as large as those seen in the peptidyl transferase assay. Further, differences in the conditions used for the footprinting and these peptidyl transferase assays lend a note of caution to direct, quantitative comparisons (in particular, the use of methionine versus phenylalanine, and the requirement for sparsomycin in the fragment footprinting experiments). These considerations notwithstanding, the qualitative similarity between the func-

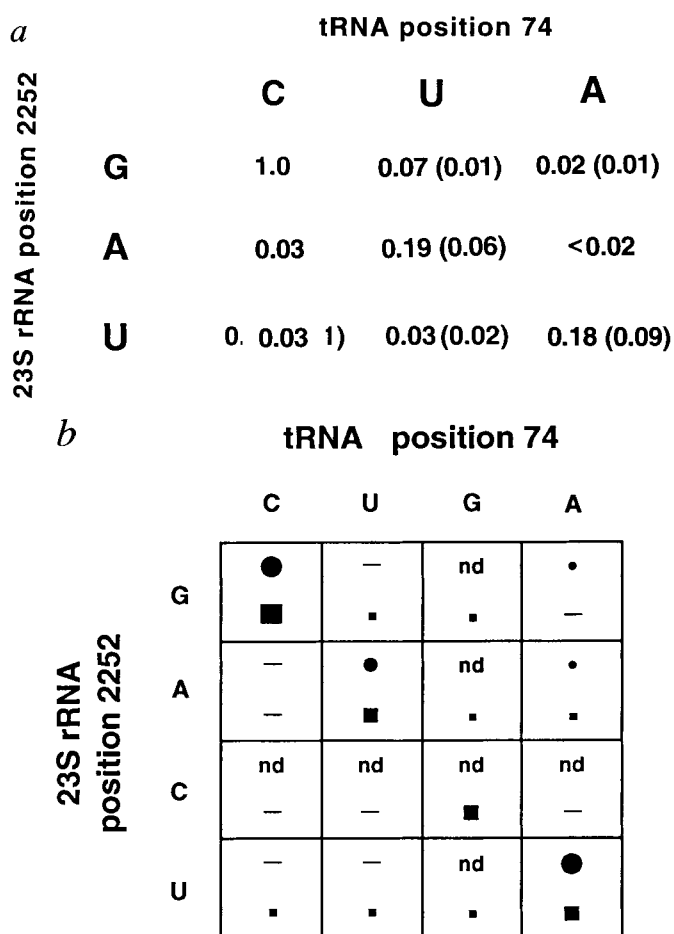


FIG. 6 a, Relative activities of chimaeric reconstitutions with various tRNA fragments. Initial rates were obtained from linear time courses of the peptidyl transferase reactions described in Fig. 5. Values were normalized to the rate of a chimaeric reconstitution mixture containing wild-type 23S rRNA sequence (G2252) in the 5' domain. Data were derived from two separate experiments (standard deviation in parentheses). b, Summary of interactions between mutant and wild-type 23S rRNAs with mutant and wild-type tRNAs. The identity of the base at tRNA position 74 is indicated at the top, and that of the base at 23S rRNA position 2252 is shown at the left. Circles indicate protection of U2585 from CMCT using the oligonucleotide assay (Fig. 3), and squares indicate protection of G2253 from kethoxal using the tRNA assay (Fig. 4). Extent of protection is indicated by the size of symbol: large symbols, 80–100% protection; medium symbols, 60–80% protection; small symbols, 20–60% protection; dashes, no detectable protection; nd, not determined.

tional and structural data emphasizes the general importance of this Watson–Crick interaction between G2252 of 23S rRNA and C74 of tRNA in the P site of the ribosome.

Conclusions

In this study we tested the effect of mutation of the two universally conserved tRNA-protected bases G2252 and G2253 of 23S rRNA on interaction of the acceptor end of tRNA with the peptidyl transferase P site. We used chemical probing in conjunction with allele-specific primer extension^{18,20} and a chimaeric reconstitution approach allowing for the direct assessment of the peptidyl transferase activity of mutant ribosomes. Suppression of mutations at position 2252 by compensatory mutations at position 74 of tRNA (summarized in Fig. 6) in these complementary assays shows that interaction of the CCA end of tRNA with the 50S P site involves Watson–Crick base pairing between positions C74 of tRNA and G2252 of 23S rRNA. This is the first demonstration of a specific base–base interaction between tRNA and rRNA. The role of this interaction is probably to position the acceptor end of peptidyl-tRNA in the peptidyl transferase site. These findings establish a direct functional role for 23S rRNA in protein synthesis.

Mutation of the universally conserved base G2252 of 23S rRNA to A, C or U confers a dominant lethal phenotype²¹ (R.R.S., unpublished data). Sedimentation analysis showed that mutant 23S rRNA is incorporated into 50S ribosomal subunits and 70S ribosomes, but is underrepresented in polysomes²¹ (R.R.S., unpublished data). These results suggest that the mutation affects one or more steps of the elongation phase of protein synthesis. Impairment of peptidyl transferase activity could impede the rate of movement of ribosomes along the mRNA, limiting the rate of translation by wild-type as well as mutant ribosomes in polysomes, thereby exerting a dominant effect.

The directly adjoining bases, C75 in tRNA and G2253 in 23S rRNA, which are also universally conserved, fail to exhibit such a Watson–Crick relationship. Perhaps related to this is the observation that mutations at G2253 confer a significantly less severe, recessive phenotype. Nevertheless, both bases are protected from chemical probes upon binding of tRNA to the 50S P site^{9,15,16}, and mutation of C75 disrupts interaction of the 3' end of tRNA

or the oligonucleotide fragment with the 50S P site (Fig. 3). An exception is the mutant tRNA bearing a U at position 75, which appears to interact with wild-type ribosomes and with G2253A mutant ribosomes nearly as well as wild-type tRNA (Fig. 4, WT, G2253A; cf. CCA and CUA lanes). Although the latter result suggests a possible purine–pyrimidine relationship between C75 and G2253, this is not supported by the results of the oligonucleotide binding experiments. One possible interpretation is that the stereochemistry of the peptidyl transferase site can only accommodate a purine (2253)–pyrimidine (75) juxtaposition, and that this is supported for non-G–C combinations by the stable binding of full-length tRNA. Another possibility is that C75 interacts by hydrogen bonding to the sugar–phosphate backbone of 23S rRNA or to a ribosomal protein. A further alternative is that a C (or U) may be required at this position to stabilize the conformation of the CCA terminus to promote binding to the 50S P site. However, the observed protection of C75 from dimethyl sulphate attack in P-site tRNA suggests that this base does interact in some way with the ribosome. Similarly, the universal conservation of G2253 and its protection from kethoxal by the CCA end of P-site tRNA^{9,10} are also unexplained. One possibility is that the backbone of the CCA end of tRNA is responsible for its protection. Here we can rule out interactions with moieties outside the CC sequence itself, because only the CCA sequence, in addition to the peptidyl group, is required for binding¹⁴, and because G2252 and G2253 are protected even when the peptidyl moiety and 3' terminal adenosine are absent⁹.

The possibility that G2251, the 5'-adjacent universally conserved guanine, might be involved in pairing with C75 was also tested using the approaches described here (R.G., R.R.S. and H.F.N., unpublished data). No evidence was found to support a simple Watson–Crick interaction between these two residues.

Finally, the other conserved nucleotide at the 3' terminus of tRNA, A76, is required for protection of two conserved nucleotides, U2506 and U2585 in 23S rRNA⁹. Either of these bases would be an obvious candidate for interaction with A76, by either Watson–Crick or Hoogsteen base pairing. We are currently testing these possibilities by approaches similar to those described here. □

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Formation of 'bullets' by hydrodynamical instabilities in stellar outflows

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IMAGES of young stars¹ and supernova remnants² often reveal small, high-density knots of material which are interpreted as 'bullets' ejected by the source and propagating at supersonic speeds into the surrounding interstellar medium. But it is unclear how these bullets could be created and accelerated without disrupting their structure. An alternative interpretation of these features is that they condense *in situ* in high-velocity stellar winds as a result of hydrodynamical instabilities—such mechanisms have been proposed to explain the condensations seen in supernova ejecta³, and may also operate in planetary nebulae. Here we show that similar processes can also form bullets in the poorly collimated winds from young stars. We therefore suggest that bullets associated with outflow sources should not, in general, be ascribed to explosive events at the source; rather, they form as a direct result of the interaction between the outflowing gas and the surrounding medium.

Recent dramatic infrared observations¹ of the OMC-1 star-forming region in the Orion molecular cloud have revealed a score of 'bullets', spread over a wide opening angle, which seem to emanate from a common source. It has long been thought that the powerful mass outflows^{4,5} emanating from young stars could be the source of supersonic 'bullets' which account for the observed properties of Herbig Haro (H H) objects associated with such flows⁶. Indeed, detailed hydrodynamical calculations of the evolution of highly collimated protostellar outflows (that is, protostellar jets) have shown that radiative cooling in the shocked jet and ambient gas leads to the formation of dense shells of material that are easily fragmented^{7,8}. Observations further reveal that the jets are highly time-variable⁹, which can induce further fragmentation^{10,11}; thus the identification of long, linear strings of H-H objects in protostellar jets with bullets formed by fragmentation seems well established. But in the images obtained by Allen and Burton¹, the bullets are spread over such a wide opening angle that they cannot all be associated with a tightly collimated jet; the authors therefore concluded that the bullets were ejected explosively by an event near the star itself. We suggest an alternative interpretation in which the bullets were formed by the fragmentation of the dense shell swept up by a time-variable and poorly collimated stellar wind.

In the Orion image, the trajectories of the bullets observed by Allen and Burton indicate an ejection centre within 5 arcsec of two luminous infrared sources in the Orion nebula: the Becklin-Neugebauer (BN) object and IRc2. BN is the dominant source at near-infrared wavelengths with a total luminosity of $(1-2) \times 10^4 L_{\odot}$ (ref. 12), whereas IRc2 is the dominant far-infrared source and has long been thought to be the primary luminosity source ($L \approx 10^5 L_{\odot}$) in the region¹³. Both objects are thought to be the sources of poorly collimated outflows^{13,14}, although there is still controversy over identification of the main outflow source in this region. Given that outflows from many low-luminosity protostars are known to be time-variable⁵, and that outflows from some high-luminosity young stars show evidence for variability¹⁵, it is quite possible that the outflow associated with the high-luminosity sources in OMC-1 is also variable, although there is little evidence to support this (other than the images of Allen and Burton).

Just as in the evolution of a supernova shell¹⁶, the bubble inflated by the stellar outflow evolves through four distinct phases¹⁷, the longest-lived of which is the 'snowplough' phase in which radiative cooling in the swept-up ambient gas causes it

to collapse into a thin shell. The stability of this shell is of particular interest here. For a steady outflow expanding into a uniform ambient medium, the shell decelerates (that is, $d^2R_s/dt^2 < 0$, where R_s is the radius of the shell). Each parcel of gas in the outflow feels an effective gravity that is directed outwards; thus the shell is stably stratified. But it can be shown^{18,19} that if the mechanical luminosity of the wind L_w is the power-law in time $L_w \propto t^m$, and the density in the ambient medium ρ is a decreasing power law in distance $\rho \propto r^{-n}$, then the shell will accelerate ($d^2R_s/dt^2 > 0$) if $n + m > 2$. Acceleration of the shell is Rayleigh-Taylor (R-T)-unstable, and leads to fragmentation into knots and filaments. The fragmentation of a stellar wind shell due to acceleration caused by a steep ($n > 2$) density gradient is thought to be important in, for example, the evolution of superbubbles evacuated by OB associations as they blow out of the galactic disk²⁰ and starburst galaxies such as M82 (ref. 21). Here we propose that time variability in the mass loss rate or velocity of the outflow (similar to the variability inferred by the presence of knots in stellar jets⁵) is responsible for acceleration, and therefore fragmentation into bullets, of the dense shell swept up by the wind from IRc2.

To test this hypothesis quantitatively, we have performed two-dimensional, time-dependent hydrodynamical simulations of the evolution of a variable stellar wind. To evolve the equations of hydrodynamics, we use an algorithm based on the piecewise parabolic method (PPM). Because the cooling time in the bullets (~ 1 year) is very much shorter than the dynamical time ($\sim 1,000$ years), we treat the gas as being isothermal rather than following the radiative cooling in detail. The use of an isothermal equation of state will preclude some interesting dynamical behaviour in the shell, such as the dynamical overstability present in the radiative shocks that bound the shell²². This overstability can introduce small amplitude warps into the shape of the shell, but it will not fragment the shell into bullets and is therefore not central to the effects studied here. The equations of hydrodynamics are solved in spherical polar coordinates in the r θ plane with a resolution of 200×100 grid points and with the source of the outflow at $r = 0$. The wind is introduced as a boundary condition at the inner edge of the grid at $r = 0.05L$, where L is a fiducial length. Instability in the shell swept up by the wind is seeded by density fluctuations in the ambient molecular gas that initially surrounds the central source. We perturb the density in the ambient gas in a narrow region near the inner boundary using two cosine functions of amplitude $\delta\rho/\rho_0 = 0.1$ and random phase, where ρ_0 is the fiducial density in the circumstellar gas. The stellar wind initially has a velocity of Mach 10 and a density of ρ_0 . We allow the wind to expand for 0.1 sound-crossing times, and then increase the wind velocity to Mach 100 for a brief interval while keeping the loss mass rate constant. When the dense shell swept up by the initial slow phase of the stellar wind is struck by the high-velocity impulse, it is accelerated sharply and is strongly R-T unstable and is fragmented. To scale the calculations to the conditions in OMC-1, we set the sound speed to 20 km s^{-1} (which, because we use an isothermal equation of state, is constant everywhere) and the characteristic length scale $L = 3 \times 10^{17} \text{ cm}$, giving a sound-crossing time of 5,000 years. The fiducial density is chosen by setting the particle density in the wind to $n_0 = \rho_0/\bar{m} = 10^3 \text{ cm}^{-3}$ (where $\bar{m}$ is the mean mass per particle), giving a mass loss rate in the wind of $10^{-6} M_{\odot} \text{ yr}^{-1}$. This scaling also implies that the fast wind has a terminal velocity of $2,000 \text{ km s}^{-1}$. Such high values are not uncommon in winds from massive stars²³. Moreover, recent observations¹⁵ of radio jets associated with deeply embedded protostars indicate outflow velocities as high as $600-1,400 \text{ km s}^{-1}$ in these sources. In any case, the dynamical process that leads to fragmentation is primarily sensitive to the ram pressure of the wind, and thus slower winds with higher mass loss rates give similar results (we have checked this using a fast wind with a terminal velocity one-half of that used here).

Figure 1 contains several snapshots of the density at four instants during the evolution. Note that the snapshots are not equally spaced in time. A linear colour map is used, with black denoting the highest densities and white the lowest. In the first frame, at $t = 250$ years, the dense shell formed by the low-velocity wind is evident. In the second frame, at $t = 610$ years, the high-velocity wind has been turned on, and it forms a second shell interior to the first as it sweeps up the slow wind material. The outer shell contains substructure introduced by the density perturbations in the ambient gas, which excite the thin-shell instability described by Vishniac and recently studied in the non-linear regime²⁴, but this structure does not resemble the bullets observed in OMC-1. By the third frame, at $t = 720$ years, the high-velocity shell has collided with the outer shell, and the brief impulse that accelerates the shell leads to fragmentation. We note that the structures observed in this frame bear a striking resemblance to those presented in hydrodynamical studies of the evolution of circumstellar gas around massive post-main-sequence stars²⁵, which undergo a similar evolutionary history of a fast wind that sweeps up slower circumstellar material. In the last frame, at $t = 1,000$ years, well separated, dense fragments propagate nearly ballistically into the ambient gas, leaving narrow cones associated with their bow shocks. For the scaling appropriate to OMC-1, the bullets propagate at about 400 km s^{-1} and contain $10^{-6} M_{\odot}$ of gas shortly after the fast wind collides with the slow shell, in good agreement with the values obtained by Allen and Burton. The knots are strongly decelerated, however, so that the average speed at $t = 1,000$ years is only about 200 km s^{-1} . Much of this deceleration is caused by our assumption of a uniform ambient medium. If the density of the ambient gas decreases with the distance from the source, as

is likely in star-forming regions, the knots will not be decelerated as strongly. The spacing of the knots is determined in part by the scale of the initial perturbations in the ambient gas. We find that if the spectrum of initial perturbations is resolved, the number and spacing of knots produced in our simulations does not change with increasing numerical resolution. On the other hand, because in the linear phase of the R-T instability perturbations with the highest wavenumbers grow fastest, if smaller-scale perturbations in the ambient gas are introduced initially, a greater number of more closely spaced knots can be produced.

Detailed modelling of the emission spectra of the bullets observed in our simulations would allow fruitful comparison with the observations²⁶. However, such modelling would require a more sophisticated treatment of the ionization, recombination and cooling of the gas than is possible with the isothermal models presented here. The fragmentation of bullets observed in our simulations is driven by the large density contrast between the cooling shell and the wind. Given that the cooling time of the gas is much shorter than the dynamical time, the formation of a dense shell is unavoidable; thus we do not expect a more realistic treatment of the microphysics of the gas to alter the fragmentation into bullets. The observed kinematics of the bullets in OMC-1 are consistent with bow shocks. Our model predicts that in the bright emission region near the source of the bullets, the observed kinematics will be complex, owing not only to the interaction of the bow shocks from the bullets, but also to the impact of the stellar wind.

We expect the hydrodynamical processes that lead to the formation of bullets studied here to be present in both high- and low-luminosity protostellar outflow sources provided that strong radiative cooling allows the swept-up gas to collect in a thin

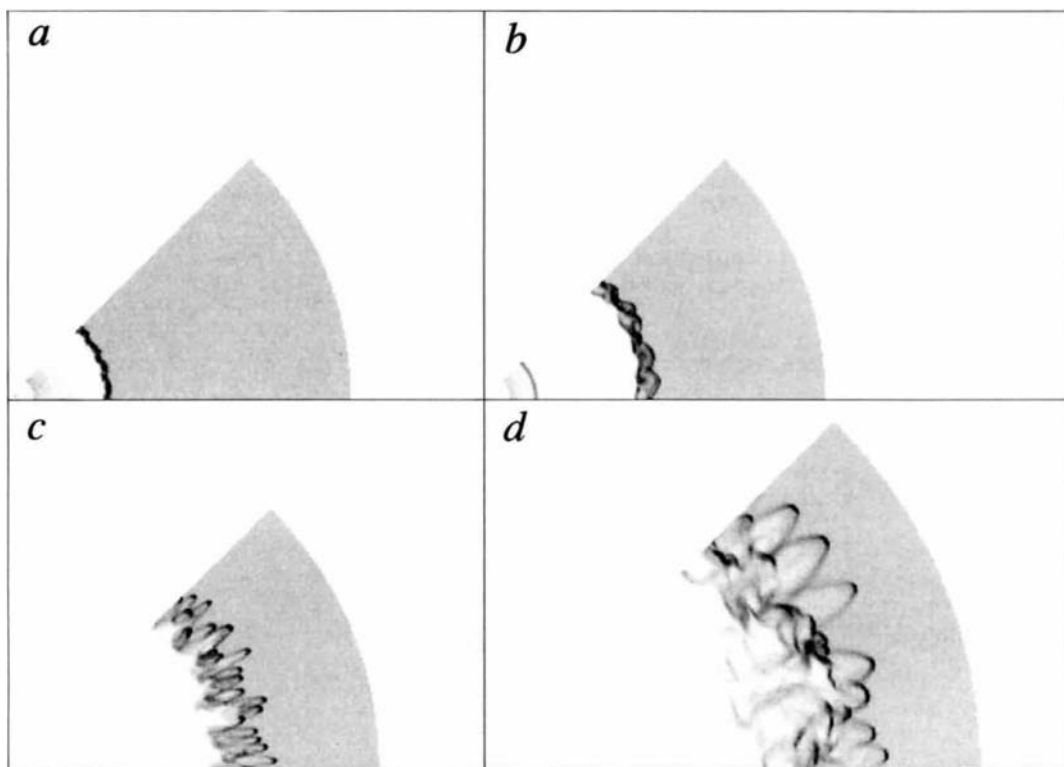


FIG. 1 Snapshots of the density taken at four unequally spaced times in the evolution of a variable stellar wind. To approximate the effects of radiative cooling, an isothermal equation of state is used. In *a* ($t = 250$ yrs), a steady 200 km s^{-1} wind with a mass loss rate of $10^{-6} M_{\odot} \text{ yr}^{-1}$ has swept up a dense expanding shell. Small-amplitude perturbations of the density in the ambient gas have excited a thin-shell instability, causing the shell to become knotty. In *b* ($t = 610$ yr), the shell has become more fragmented, but still does not resemble 'bullets' in

the ambient gas. At the same time, increasing the wind speed to 2000 km s^{-1} has resulted in the formation of a thin, smooth, second shell consisting of swept-up slow wind material near the origin. By *c* ($t = 720$ yr), this second shell collides with the first, fragmenting it into bullets. The wind speed from the central source is then returned to 200 km s^{-1} and *d* ($t = 1,000$ yr) shows the structure of the bullets as they propagate ballistically into the ambient gas.

shell. For example, the morphology of structures observed in the western component of the optical outflow from Cepheus A (the GGD37 complex²⁷) is similar to the structures observed in Fig. 1. The fragmentation of HH2 into distinct clumps²⁸ could also have been caused by processes similar to those studied here. In many cases (such as HH2) the acceleration of the shell that leads to fragmentation into clumps might not necessarily be caused by variability of the source, but might be caused by very steep (that is, $n > 2$) density gradients associated with the edges of molecular clouds.

The hydrodynamical processes that lead to fragmentation into bullets in OMC-1 are not confined merely to outflows from young stars. For example, there is a wide variety of hydrodynamical instabilities in the evolution of a supernova shell that can lead to fragmentation and the formation of bullets in these systems as well. The shell of ambient material swept up by the blast wave is subject to a radiative overstability²², the thin-shell instability²⁴, and to the R-T instability in the early phases of its evolution²⁹. Moreover, at the centre of the remnant, the ejecta arising from the stellar envelope is R-T-unstable during the acceleration phase³⁰ and is subject to convective instabilities³¹, which both mix the ejecta and cause it to become clumpy. Finally, if a pulsar is formed in the supernova event, the expansion of a synchrotron nebula created by the spin-down of the pulsar can lead to acceleration of the ejecta during the early phase of evolution of the remnant. Such acceleration is yet another mechanism for producing fragmentation. In fact, the dense and long fingers of ejecta observed inside the Crab supernova remnant by the Hubble Space Telescope (HST) are best interpreted as being caused by a magnetic R-T instability as the ejecta is accelerated by the magnetized synchrotron nebula inflated by the Crab pulsar³. The velocity of the knots associated with the fingers in the Crab filaments is about $2,000 \text{ km s}^{-1}$. Using the Sedov-Taylor similarity solution¹⁶ to describe the growth of the blast wave, and assuming an ambient density of $N_H = 1 \text{ cm}^{-3}$, we find that the knots will strike the blast wave in 3,000 years assuming that they maintain their current velocity. Remarkably, recent X-ray observations of the Vela supernova remnant² have revealed a structure identified as a bullet that has punctured the blast wave and is now propagating into the ambient ISM. The observed velocity of the bullet and the age of the Vela remnant are in good agreement with the time calculated above for knots of ejecta to impact the blast wave. Thus we propose that processes similar to those observed in the Crab are responsible for forming the bullet discovered in Vela.

Over long timescales, variations in the mass loss rate in winds associated with massive stars can produce structures in the circumstellar gas that resemble bullets. Detailed two-dimensional hydrodynamical calculations²⁵ of a time-variable wind produced by evolution of the central star from the main sequence through the luminous blue variable stage and into the onset of the Wolf-Rayet stage are strikingly similar to the results presented here for protostellar winds.

New observations of planetary nebula indicate that these outflows may also form dense knots. For example, recent HST observations of NGC6543 reveal a complex system of knots and filaments in this source³². The observed line ratios indicate that the density, temperature and ionization balance are highly variable in the outflow, and it is likely that hydrodynamical processes have led to the clumpy nature of the nebula. Therefore, although supersonic bullets are associated with a very wide variety of astrophysical outflow sources, it would appear that similar hydrodynamical processes are responsible for their formation in each case. $\square$

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White-light velocimetry

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RADIATION reflected from a moving object experiences a Doppler shift in its frequency. This basic phenomenon underlies practical interferometric techniques for remote velocity measurement used widely in meteorology and law enforcement, as well as in medicine¹ (using acoustic waves) and other fields of science and engineering^{2–5}. Existing velocimetric techniques use coherent quasi-monochromatic sources of illumination, which, for optical applications, generally limits practical sources to lasers operating in single-frequency mode. Such sources are not, however, sufficiently powerful for applications where simultaneous velocity measurements at many points on a target are desired. Here we describe a technique for remote velocity measurement that uses broadband incoherent illumination. The viability of this technique is demonstrated by measuring the velocity of a target moving at 16 m s^{-1} using white light from an incandescent source. Powerful, compact and inexpensive radiation sources (such as flash and arc lamps, or lasers operating at several wavelengths) can now be exploited for high-power applications of remote-target velocimetry.

The VISAR^{2–5} velocimeter (velocity interferometer system for any reflector) is an optical interferometer having a fixed delay τ between its arms. This delay converts small Doppler shifts into fringe shifts in the interferometer output. Let the delay be specified by the distance ($c\tau$) that light travels in that time, where c is the velocity of light in vacuum. The idealized velocity per fringe proportionality^{2,3} (η) of a VISAR is

$$\eta = c\lambda/2c\tau \quad (1)$$

where λ is the average wavelength of light. For example, to measure highway velocities in green light with $\eta = 10 \text{ m s}^{-1}$ requires $c\tau \approx 8 \text{ m}$. Equation (1) neglects dispersion³ in the glass optics inside the interferometer and assumes that $v/c \ll 1$.

In previous VISARs, the coherence length (Λ) of the illumination must be as large as $c\tau$ to produce fringes with significant

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visibility. This severely restricted the kind of light source that could be used. The λ of white light ($\sim 1.5 \mu\text{m}$) was insufficient. Previously, lasers have been the only light sources used in VISARs because their coherence length could be made sufficiently long when operated in a single-frequency mode. However, in this mode the output power is low.

Because of this, typical laboratory measurements in shock physics were limited to measurement of velocity at a single point on the target. These experiments^{6,8} can measure both the structure and arrival time of a shock wave after propagating through a sample—information that can yield the equation of state (pressure against volume), the transition between elastic and plastic flow (controlled by material strength), or the presence of phase transitions^{6,7}.

Such experiments could be greatly improved if we could measure sample velocity simultaneously at more than one point. For example, multipoint velocimetry on a wedged sample could show the evolution of the shockwave profile against propagation depth. Moreover, measurements of velocity across a line or an area over a target could diagnose the chaotic motion of an accelerated and spatially perturbed interface between materials of unequal densities (called Rayleigh–Taylor⁹ or Richtmyer–Meshkov¹⁰ instability, depending on the details of the acceleration). This issue is relevant to implosions used in laser fusion.

Previously, multipoint or line¹¹ VISAR velocimetry has been very expensive. Lasers with sufficiently long coherence length were relatively weak, and required optical amplifiers to illuminate more than a single point. Velocimetry over a surface, or of a remote object through a telescope in the field, demands orders of magnitude more power, particularly if scale coherence lengths of many metres are needed (such as needed for resolving $\sim 10 \text{ m s}^{-1}$ velocities).

We have developed a simple and generic method (Fig. 1) of preparing any wave source, regardless of its initial coherence, to illuminate a velocity interferometric experiment. A coherent echo is imprinted on the illuminating light by an interferometer (denoted the source interferometer) having a delay τ_s . The reflected light from the target is observed through a second interferometer (denoted the detecting interferometer) having delay

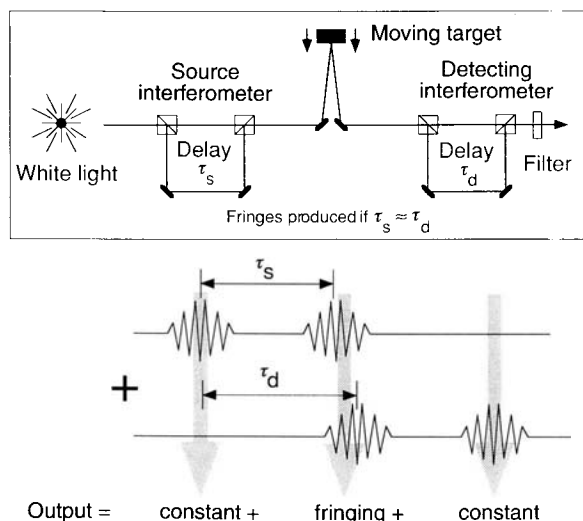


FIG. 1. White-light velocimeter concept. Two interferometers before and after the target have similar delays τ_s and τ_d . White light is a series of independent wave packets. The first interferometer splits each wave packet into two identical packets; the second does the same to create a total of four. Two of these packets will overlap if $\tau_s \approx \tau_d$, producing fringes. The other packets contribute constant intensity. The target's velocity will change the apparent τ_s owing to the Doppler effect, causing a fringe shift. The wave-packet length is inversely proportional to system bandwidth, represented by the filter.

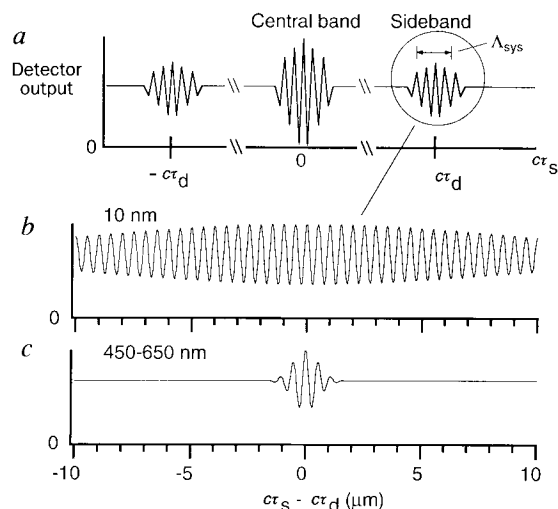


FIG. 2. White-light velocimeter output plotted against source-interferometer delay length (τ_s), for a stationary target. Both source and detecting interferometers are of the Michelson type. A moving target Doppler-shifts the apparent τ_s by $\Delta(\tau_s) = -(2v/c)(\tau_s)$. a, Diagram of global behaviour: sidebands have the same shape and width as the central band, but lower amplitude. Their width, the system coherence length (Λ_{sys}), is inversely proportional to the bandwidth of the system spectra. b, Calculated detail for 495–505-nm Gaussian system spectra. c, As b, but for 450–650 nm bandwidth.

τ_d . Partial fringes result when τ_s and τ_d are within a coherence length of each other. Target velocity causes the apparent value of τ_s to change owing to the slight Doppler scaling of the spectrum reflected from the target, producing a fringe shift.

Double-interferometer arrangements using short-coherence-length light have been used previously in communication¹² and to multiplex serial fibre optic sensors¹³, and to measure target motion where the target is internal to one of the interferometers^{14–16}. But we believe we are the first to apply this concept to moving targets external to an interferometer. This allows the fringe phase to be independent of target distance, surface roughness, and the scattering and dispersive properties of the interposed medium. A single-interferometer technique by Geindre *et al.*¹⁷ measures high-velocity (100 km s^{-1}) plasma by illuminating the plasma with two subpicosecond pulses separated by $\sim 1 \text{ ps}$, then dispersing the reflected light with a grating. Their Doppler shift is resolvable by a grating rather than a second interferometer because it is so large.

In general, there is no restriction on the design of either interferometer. However, interpretation of the fringe shift is most straightforward when the detecting interferometer is of a Michelson type. In our demonstration, the same optical system performs the functions of both the source and the detecting interferometers. This makes alignment much easier, because $\tau_s \approx \tau_d$ automatically. However, in applications where the illumination is very much brighter than the light reflected from the target, the source and detecting interferometers should be separate to isolate the glare of illuminating light from shared optics.

The Doppler effect scales the spectrum of the source interferometer by a factor $(1 + 2v/c)$. Because this spectrum's periodicity is controlled by τ_s , this is equivalent to scaling the apparent value of τ_s to $\tau_s/(1 + 2v/c)$. Hence, there is an apparent shift in τ_s of

$$\Delta(\tau_s) = \frac{c\tau_s}{1 + 2v/c} - c\tau_s \approx -\left(\frac{2v}{c}\right)c\tau_s \quad (2)$$

Figure 2 shows the calculated fringes plotted against $c\tau_s$, for Michelson source and detecting interferometers and a stationary

target. Fringe behaviour with velocity is obtained from these curves by shifting the initial value of $c\tau_s$ by the amount $\Delta(c\tau_s)$ given by equation (2).

The fringe visibility is defined as $(I_{\max} - I_{\min}) / (I_{\max} + I_{\min})$, where $I_{\max}$ and $I_{\min}$ are local maximum and minimum output intensities. As Fig. 2 shows, to obtain significant fringe visibility one needs $c\tau_s \approx c\tau_d$, within a distance we call the system coherence length Λ_{sys} , where $\Delta\lambda$ is the bandwidth of the entire system including the detector. For Michelson source and detecting interferometers, the fringe visibility does not exceed 50%. This is not a practical difficulty: because the two outputs of the detecting interferometer are of opposite phase, if they are both recorded they can be subtracted numerically to cancel the non-fringing signal portion. Also, using a Fabry-Perot source interferometer can increase fringe visibility arbitrarily at the expense of decreased absolute signal amplitude, depending on the Fabry-Perot mirror reflectivity R .

We demonstrate white-light velocity interferometry by photographing the fringes from a 16 m s^{-1} moving target with incandescent light. Velocities of this order are more challenging to measure than velocities of order 1 km s^{-1} because of the smaller Doppler shifts. These are resolved by larger values of $c\tau$, which in turn require larger-diameter optics for a given angular field of view. Figure 3 describes our white-light velocimeter having $c\tau_s \approx c\tau_d = 4 \text{ m}$ and $\eta \approx 19 \text{ m s}^{-1}$ per fringe. By retro-reflecting the light from the target the functions of the source and detecting Michelson interferometers are accomplished by the same optics. This automatically ensures that $\tau_d \approx \tau_s$. The interferometer mirrors are misaligned slightly to produce a ladder of fringes to make them more apparent.

An interferometer delay of 4 m is accomplished by the optics enclosed by the dashed box in Fig. 3. These create a virtual plane mirror, from a ray-tracing aspect, but a delay of 4 m in the time of flight of a wave packet. The target was an electric fan with reflective tape on its blades. The beam intercepted the blade from 45 to 54 mm radius, at an angle to the rotation plane of 20.5° . The frequency of rotation was determined stroboscopically to be 53.8 Hz. This yields a velocity component of $15.7 \pm 0.3 \text{ m s}^{-1}$ parallel to the light. (The uncertainty is in the average fan radius struck by the beam.)

Figure 4 compares photographs of fringes from the stationary and the moving target. The fringe pattern has visibly shifted

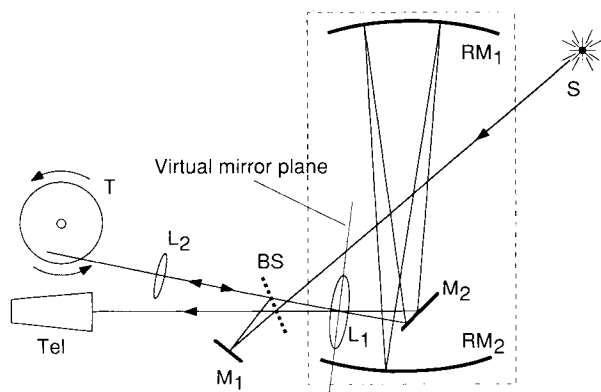


FIG. 3 White-light velocimeter with 4-m delay and $\eta \approx 10 \text{ m s}^{-1}$ per fringe. S, incandescent lamp; T, target is fan with reflective tape on blades; Tel, telescope or camera; BS, 50% beamsplitter; M_1 , M_2 , plane mirrors; RM_1 , RM_2 , mirrors with a 1-m radius of curvature separated by 1 m; L_1 , 1-m focal-length lens. The optical system in the dashed box acts as virtual plane mirror at a position superimposed on the reflective image of M_1 about BS. L_2 , lens imaging the target to the virtual mirror plane. Because light retro-reflects from the target, the same optics perform the functions of the source and the detecting interferometers. The other interferometer output (not used here) travels toward the source and is accessible by inserting a beamsplitter between BS and S.

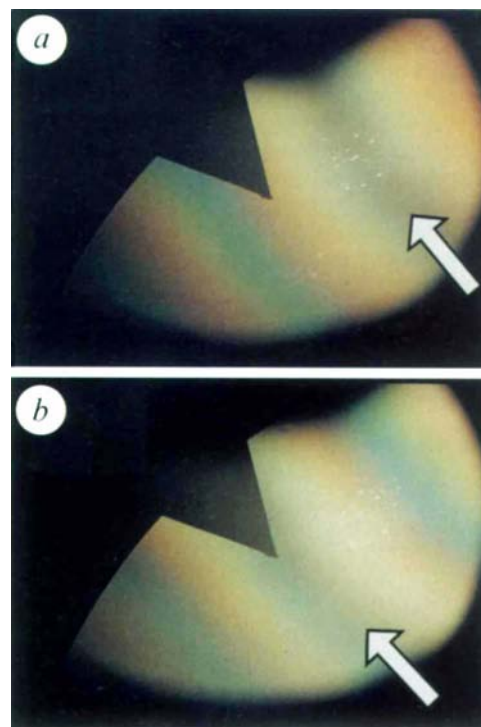


FIG. 4 White-light fringes of the target. a, Stationary target; b, target moving at 15.7 m s^{-1} . Triangle silhouette at image plane (RM_1) provides a reference location. The dark central fringe (white arrows) can be distinguished by its colourless grey appearance and symmetrical placement relative to the coloured fringes, particularly the red fringes. From a to b the central fringe has shifted toward the triangle. When viewed in 500-nm light the shift is 0.85 fringe. The bright flecks are dust specks on RM_1 illuminated by the source beam.

toward the reference triangle owing to target velocity. Observation through a 40-nm-wide 500-nm bandpass filter shows the fringe shift to be 0.85 ± 0.07 . For $c\tau = 4 \text{ m}$ and $\lambda = 500 \text{ nm}$, $\eta = 18.7 \text{ m s}^{-1}$ per fringe from equation (1). This yields an interferometrically measured velocity of $15.9 \pm 1 \text{ m s}^{-1}$. This agrees with the $15.7 \pm 0.3 \text{ m s}^{-1}$ measured stroboscopically.

The significance of this demonstration is twofold. First, we observe fringes from an incoherent source with an interferometer delay greatly exceeding the $\sim 1.5 \mu\text{m}$ coherence length of white light. Second, we demonstrate a fringe shift with target velocity, and this is a low velocity (by shock physics standards) measured remotely with easily obtained optics and an inexpensive light source.

Because a conventional VISAR uses monochromatic illumination, a discontinuous velocity jump will cause an ambiguity in the fringe shift by an integer. This because fringes are periodic. But in the white-light velocimeter, by recording fringe shifts for different colours separately, the velocity can be uniquely determined. One implementation of this is to disperse the velocimeter output by a grating and record the spectrum versus time by a multichannel detector or streak camera. The number of fringes across the streak record in the wavelength direction scales with the velocity, and therefore the velocity skip across the shock is determinate.

Our demonstration measured the velocity of a small area of the fan blade because of the limited angular field of view afforded by the optics we had on hand. We are constructing a new velocimeter with separate source and detecting interferometers, which will allow much brighter illumination with a wider field of view.

The white-light velocimeter should lead to many new applications of velocimetry made practical by inexpensive or powerful incoherent sources. For example, a fluid's velocity field could be measured over a large area on a microsecond or nanosecond

timescale to high precision. This could aid in the study of turbulence. The ultimate spatial resolution would be superior to conventional fluid Doppler velocimetry¹⁸ (where particles cross a standing fringe pattern formed by intersecting laser beams) because the speckle of white light is negligibly small, and because this fluid technique is limited to measurement at a single point. Interferometers implemented with single-mode optical fibre having kilometre delays could measure velocities of order 1 mm s^{-1} , although for a single target point. Such velocity sensitivity has already been demonstrated in a fibre-optic Sagnac¹⁶ interferometer design with a 200-m delay. In our design, the target is external to the interferometer, which allows practical velocimetry of remote targets out of doors. The principle can be applied to other wave phenomena such as microwaves and ultrasound. □

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Fabrication of hollow porous shells of calcium carbonate from self-organizing media

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A RICH variety of elaborate microscopic skeletal structures composed of inorganic materials are produced in nature¹. Such complex, three-dimensional structures, if produced synthetically, could find important applications as light-weight ceramics, catalyst supports, biomedical implants and robust membranes for high-temperature separation technology. Here we describe a method for synthesizing hollow porous shells of crystalline calcium carbonate (aragonite) that resemble the coccospheres of certain marine algae. We show that thin cellular frameworks of either mesoporous or macroporous aragonite can be formed from oil–water–surfactant microemulsions supersaturated with calcium bicarbonate, with the pore size determined by the relative concentrations of water and oil. Using micrometre-sized polystyrene beads as the substrate for the microemulsion, hollow spherical shells of the honeycomb architecture can be produced. We propose that these cellular frameworks originate from rapid mineralization of aragonite, with a self-organized foam of oil droplets acting as a structural template, and suggest that similar processes could be of general importance in materials chemistry.

We are interested in discovering chemical routes to the fabrication of inorganic materials with organized hierarchical structure and form, analogous to the exquisite biomineralized architectures fashioned by many single-celled organisms such as coccoliths and radiolarians. In this regard, the use of oil–water–surfactant mixed-phase systems as organized reaction media seems to be a promising approach. The phase behaviour and composition of these systems can be varied extensively, indicating that markedly different inorganic structures and architectures could be fabricated. For example, whereas water-rich hexagonal and cubic phases can direct the formation of mesoporous silicas², limited amounts of water in bicontinuous microemulsions can give rise to reticulated frameworks of amorphous silica³ or calcium phosphate⁴. In the latter case, the macroporous architecture consists of interlinked micrometre-sized crystals, indicating that the length scales of the original microemulsion network and inorganic ‘replica’ are incommensurate. This sug-

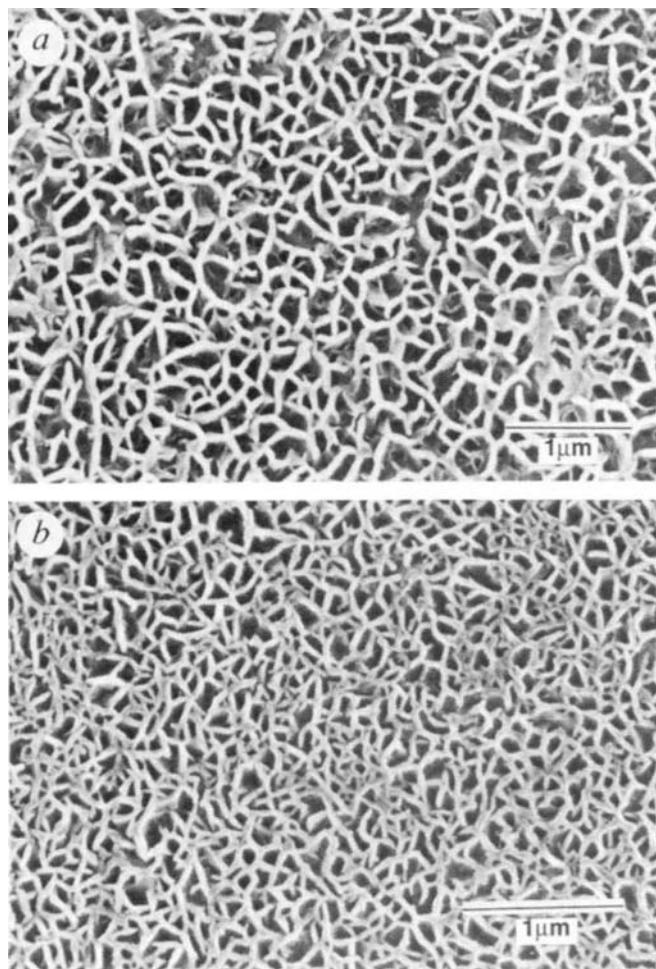


FIG. 1 Scanning electron micrographs of cellular frameworks of aragonite formed on metal substrates. a, Product from a bicontinuous microemulsion with a water:oil ratio of 1.4:1, using a tetradecane/hexadecane oil. The average pore size is 225 nm. b, Product from tetradecane-containing microemulsion with a water:oil ratio of 0.69, after heat treatment at 200 °C. The average pore size is 110 nm.

METHODS. Bicontinuous microemulsions with a water:oil ratio of 0.69 were prepared from mixtures of 0.68 g DDAB, 0.72 g tetradecane and 0.50 g of freshly prepared 5 mM Ca^{2+} /25 mM Mg^{2+} supersaturated solution (36:38:26 by weight respectively). A high-water (cubic) phase was prepared by mixing 0.64 g DDAB, 0.12 g tetradecane and 1.2 g of Ca^{2+} / Mg^{2+} solution (34:6:60 by weight, respectively). Electron microscopy was undertaken with a Jeol 1200EX electron microscope. XRD patterns were recorded on a Philips PW-1130 X-ray diffractometer fitted with a Debye–Scherrer camera and Cu K α radiation.

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gests that phase separation and a secondary mode of self-organization facilitate the construction of the microskeletal inorganic material. A related mechanism, involving the self-organization of water droplets during phase separation of CS_2 /polymer solutions, has been proposed to account for the formation of periodic honeycomb arrays of porous polystyrene films⁵.

Thin cellular frameworks of porous calcium carbonate were prepared from bicontinuous microemulsions containing the cationic quaternary ammonium surfactant didodecylmethyl ammonium bromide ($(\text{C}_{12}\text{H}_{25})_2(\text{CH}_3)_2\text{NBr}$, DDAB), tetradecane and supersaturated aqueous calcium bicarbonate solution⁴. The supersaturated solution was prepared by bubbling carbon dioxide gas at 3 l min^{-1} through an aqueous suspension of calcium carbonate (2.5 g l^{-1}) for 1–1.5 hours. The resulting supersaturated solution ($[\text{Ca}^{2+}] \approx 10\text{ mM}$) was filtered and magnesium chloride added (mole ratio of $[\text{Ca}^{2+}]:[\text{Mg}^{2+}] = 1:5$) to promote the growth of the aragonite polymorph of calcium carbonate⁶. This solution was diluted within the range of one-half to one-sixth of its original concentration by addition of distilled water, and then added dropwise to a rapidly stirred DDAB/tetradecane mixture at room temperature to give an optically clear bicontinuous microemulsion. Different compositions (water:oil ratios from 0.69:1 to 10:1) within the single-phase region were studied. In some experiments a mixed oil phase of tetradecane and hexadecane was used.

A few drops of the bicontinuous microemulsion were spread onto a metal (copper or brass) substrate, such as a scanning electron microscopy (SEM) stub, and carefully washed by horizontal immersion of the substrate into either hot chloroform at 55°C or hexane at 65°C for one or two seconds, followed by removal and evaporation of any residual hot solvent in air. This procedure was repeated four or five times until dissolution of the organic oil and surfactant was complete. A white residue was observed on the substrate surface, which was shown to be aragonite by X-ray diffraction (XRD) (observed d spacings in $\text{\AA}$: 3.39 (111), 3.27 (021), 2.70 (012), 2.48 (200), 2.37 (112), 2.33 (130), 2.18 (211), 2.10 (220), 1.97 (221), 1.88 (041), 1.81 (132), 1.74 (113), 1.72 (231)). No other calcium carbonate polymorphs, including magnesium-substituted calcite, were detected. Energy

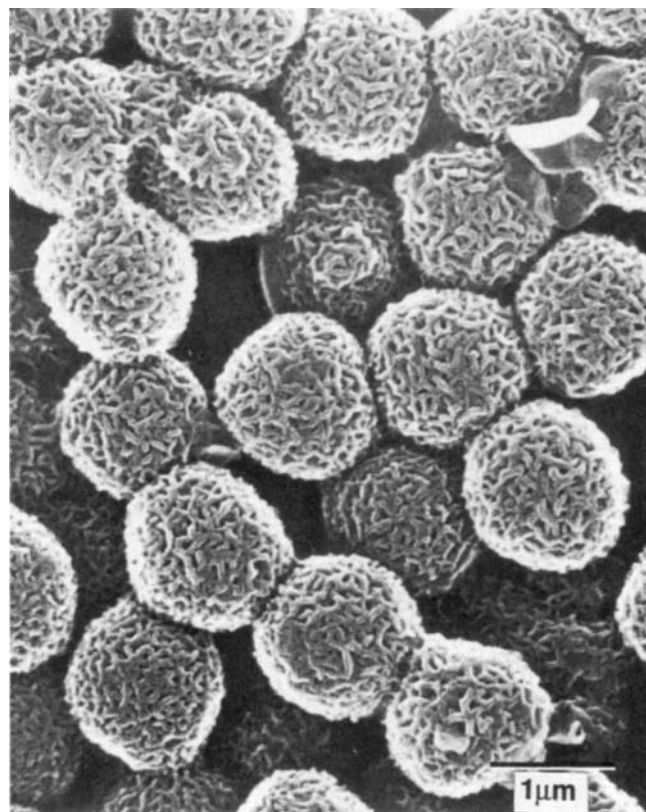


FIG. 2 Intact hollow shells of mesoporous aragonite.

dispersive X-ray analysis of washed samples detected calcium throughout the structure with some magnesium in localized regions. Examination by SEM of the aragonitic material showed the presence of a thin film of calcium carbonate consisting of a honeycomb microstructure of irregular-shaped columnar pores with interconnecting crystalline walls often at $\sim 120^\circ$ to each

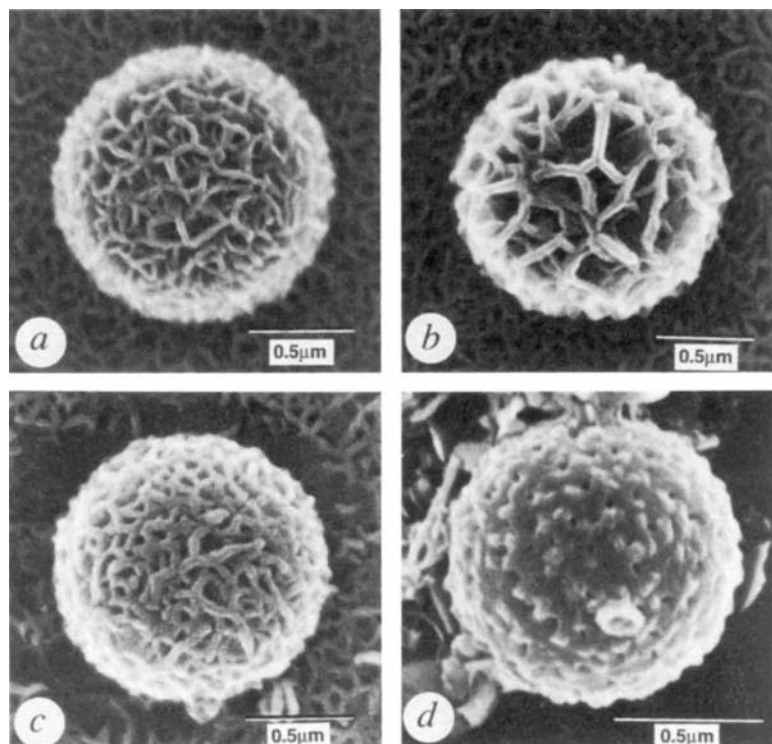


FIG. 3 a–c, High-magnification SEM images of synthetic aragonite shells showing differing textures and porosities. d, Coccosphere of the marine alga, *Thoracosphaera* (courtesy of S. A. Davis).

other (Fig. 1a, b). Heat treatment of the structure up to temperatures of 200 °C for five minutes had no significant effect on the cellular framework (Fig. 1b). Transmission electron microscopy of fractured samples showed mineral fragments that gave single-crystal electron-diffraction patterns corresponding to aragonite, consistent with the XRD data. In a few cases, fragments of intact junctions were analysed by electron diffraction and shown to consist of single-crystal blocks with outgrowths along different crystallographic directions.

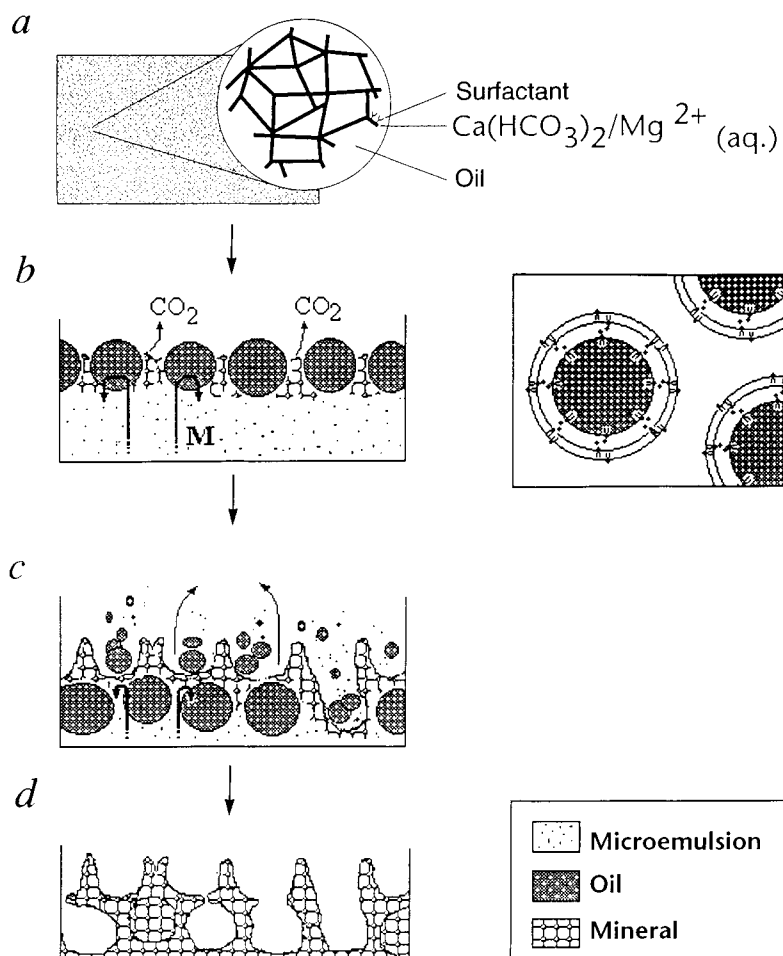
Examination of materials formed from microemulsions with different water:oil compositions showed an increase in the pore size from the mesoscopic (<50 nm) to submicrometre scale on increasing the oil content of the microemulsion. Average pore sizes between 35 nm (water:oil=10:1; wall thickness, 15 nm) and 130 nm (water:oil, 0.69:1; wall thickness, 40 nm) were achieved with tetradecane as the oil phase. A larger average pore size of 225 nm was obtained by using a mixed oil (tetradecane:hexane=2:1) at a water:oil ratio of 1.4:1 (Fig. 1).

The above processing strategy was used to fabricate hollow spherical shells of perforated calcium carbonate. Monodisperse polystyrene spheres with a uniform diameter of 1.09 µm were rendered hydrophobic by sputter-coating with a nanometre-thick layer of metallic gold. The spheres were then covered in a film of the above microemulsions and washed repeatedly in hot hexane. (Chloroform was not used because of the high solubility of the polymer in this solvent.) Removal of the surfactant and oil resulted in the formation of a continuous cellular film of aragonite over the surface of the polymer beads. Subsequent slow dissolution of the polystyrene template in a series of acetone/ethanol solvents of increasing acetone concentration (from

initially 66% (v/v) to 100%), followed by heat treatment of the mineral shells at 400 °C for five minutes, produced intact microshells of porous aragonite (Fig. 2). The hollow shells were uniform in size, with an external diameter and wall thickness of ~1.35 µm and 125 nm, respectively. Fracturing of these shells or dissolution with dilute acid revealed the absence of any internal polymeric matrix. The pore size of the cellular wall could be controlled to some extent by modifications in the water:oil ratio of the microemulsion such that a range of different textures could be produced (Fig. 3a-c), some of which displayed a superficial resemblance to the calcium carbonate coccospheres of the marine alga *Thoracosphaera*⁷ (Fig. 3d).

The above inorganic materials exhibit order on at least three length scales. They are crystalline at the unit cell level, cellular on the mesoscopic/submicrometre scale, and either spheroidal or film-like at the micrometre and macroscopic levels, respectively. We propose the following mechanism of formation for these hierarchical structures (Fig. 4). Nucleation of aragonite is dependent on increases in the supersaturation of the aqueous phase arising from outgassing of carbon dioxide. This appears to be severely limited in the bulk microemulsion—for example, filtration of microemulsions after storage for several days or weeks using 0.2 µm nucleopore polyester filters did not reveal any organized architectures—possibly owing to the viscosity of the medium and low solubility of CO₂ in the alkane oil. By comparison, similar procedures with bicontinuous microemulsions containing supersaturated calcium phosphate solutions result in the separation of intact mineral frameworks that are formed in the bulk phase⁴. Furthermore, because the cellular framework was obtained from freshly formed microemulsions as well as from samples stored for several weeks, it seems clear

FIG. 4 Proposed mechanism of formation of thin cellular frameworks of aragonite. a, Bicontinuous microemulsion of supersaturated aqueous calcium bicarbonate and tetradecane oil with DDAB surfactant at the oil–water interface. b, Washing in hot chloroform results in phase separation and self-organization of mesoscopic oil droplets by Marangoni convection (M). Loss of CO₂ at the air–water interface results in aragonite crystallization within the continuous aqueous layer surrounding the oil droplets. Inset, a top view of the proposed arrangement, with the oil droplets stabilized by a thin soapy aqueous film distinct from the continuous phase of the supersaturated solution. c, Continued washing removes oil and surfactant, leaving the mineralized boundary walls and a second layer of organized oil droplets. d, Complete removal of the oil and surfactant and heat treatment result in a mineralized replica of the foam-like reaction media.



that construction of the architecture is specifically associated with solvent extraction of the microemulsion when mounted on metal or polymeric substrates. We suggest that the loss of oil and surfactant during solvent extraction induces microscopic phase separation such that the mixture self-organizes, possibly by Marangoni convection⁸, into close-packed oil droplets bounded by a thin continuous layer of aqueous calcium bicarbonate solution. This demixing is stabilized by surfactant molecules at the oil/water interface, which, together with the Laplace attractive force of the air/water meniscus, establishes a persistent biliquid foam. In support of this mechanism, we note that biliquid foams of oil droplets coated in aqueous soapy shells and dispersed within a continuous aqueous phase are known in a number of systems⁹, and a similar effect has been recently proposed for the preparation of porous polymer films by solvent evaporation in moist air⁵. In our system, simultaneous formation of the oil droplets (by phase separation) and supersaturated aqueous solution (by rapid CO₂ outgassing from the thin aqueous layer) results in rapid mineralization of the interstitial spaces and droplet boundary edges, which on complete removal of the alkane oil leaves an inorganic replica of the self-assembled foam. Increasing the oil : water ratio increases the pore size of the cellular structure because phase separation results in larger oil droplets within the reorganized structure.

The interplay between the rate of inorganic nucleation and foam assembly appears to be critical for the construction of the organized architecture. We have undertaken similar experiments with calcite crystallization in which we prepared the supersaturated

solution in the same way, except that no Mg²⁺ was added. In such cases, optically clear microemulsions were formed but no cellular films were observed after removal of the oil and surfactant. Instead, disorganized aggregates of calcite crystals 1–10 µm in size were identified by SEM. These results suggest that the rate of nucleation of calcite is not sufficiently high to produce a consolidated wall structure before complete removal of the oil droplets.

We consider that the microscale phase separation of reaction media might have general importance in the synthesis of inorganic materials with unusual form and hierarchical structure. A critical aspect is that the assembly of foams and other metastable phases is synchronized with the onset of materials deposition, so that the organized medium can be replicated in solid form. Further investigations involving silica, metal sulphides and mixed-metal systems are currently being undertaken to test the general applicability of using self-organizing oil/water reaction media in materials synthesis. □

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Rapid climate variability in the North Pacific Ocean during the past 95,000 years

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RECENT studies of Greenland ice cores^{1–3} and North Atlantic deep-sea sediments^{4,5} have yielded evidence for rapid changes in climate during the last glaciation and the preceding interglacial period. Similar variability has been observed in lake deposits in France⁶ and in sediments from the California margin⁷, but there are no records of rapid climate variability from the high-latitude North Pacific region. Here we present two high-resolution records of the input of ice-rafted detritus to the sub-arctic Pacific Ocean, derived from two sediment cores using a γ -ray attenuation technique that provides a measure of the ratio of biogenic opal to terrigenous material. The temporal variability of ice rafting is similar to that of the oxygen isotope record of the GRIP Greenland ice core¹, implying that the rapid climate variability of the circum-Atlantic region over the past 95,000 years could be a circumpolar phenomenon.

It has been known for a long time that North Pacific deep-sea sediments contain intervals of ice-rafted detritus (IRD) throughout the Late Pliocene and Pleistocene interval^{8–13}, but before Ocean Drilling Program (ODP) Leg 145 there were no long, high-resolution records to permit detailed climatic and palaeoceanographic reconstructions. In this study we have compared high-resolution Gamma Ray Attenuation Porosity Evaluator (GRAPE) density records from two North Pacific sites in the IRD belt with the ice-core $\delta^{18}\text{O}$ record ($\delta^{18}\text{O} = [({}^{18}\text{O}/$

${}^{16}\text{O})_{\text{sample}}/({}^{18}\text{O}/{}^{16}\text{O})_{\text{standard}}] - 1$) from Summit, Greenland (GRIP)¹. Sites 882 (50° 21.8' N, 167° 36.0' E) and 883 (51° 11.9' N, 167° 46.1' E) at water depths 3,244 m and 2,385 m, respectively, were drilled from the Detroit Seamount in the North Pacific Ocean¹⁴ (Fig. 1a).

The GRAPE used in this study was originally developed by scientists at the Marathon Oil Company¹⁵. This GRAPE instrument used by ODP measures the attenuation of γ -rays passed through the unsplit core. A computer-controlled motor-driven track moves the core slowly (in 150-cm sections) between the γ -ray source (${}^{137}\text{Cs}$) and a counter. Given the assumption of a constant mineralogical attenuation coefficient¹⁶, the γ -ray count can be related directly to wet-bulk density. In marine sediments wet-bulk density depends on porosity and on grain density. The sediment porosity is the result of several factors, such as grain size, sorting, grain shape, packing of grains, and mineralogy¹⁷. Because porosity and grain density depend on the type of the sediment, GRAPE density measurements include useful lithological information. In the past few years GRAPE has been used as a palaeoceanographic tool^{18–21}. During ODP Leg 145, GRAPE density measurements from the unsplit sections were made on board ship at ~1.5-cm intervals. Sites 882 and 883 had relatively high sedimentation rates of ~5.7 cm kyr⁻¹ during the last glacial/interglacial period. With this sedimentation rate the sampling resolution is equivalent to ~0.3 kyr, and even taking account of bioturbation the resolution is probably <1 kyr.

The cores studied here were recovered with the Advanced Piston Coring technique, which is designed to produce minimal core disturbance. To produce continuous sediment records, data from different holes from each site were spliced together. At Site 882 composite sections were made by splicing holes 882A and 882B together and at Site 883 holes 883D and 883C were spliced (Fig. 1b).

Chemical analysis shows that these sediments are predominantly composed of biogenic opal and terrigenous material. In the upper part of the sediment column (upper 10 m), carbonate content is very low (0.17 wt%) (Fig. 2d). In this sub-arctic

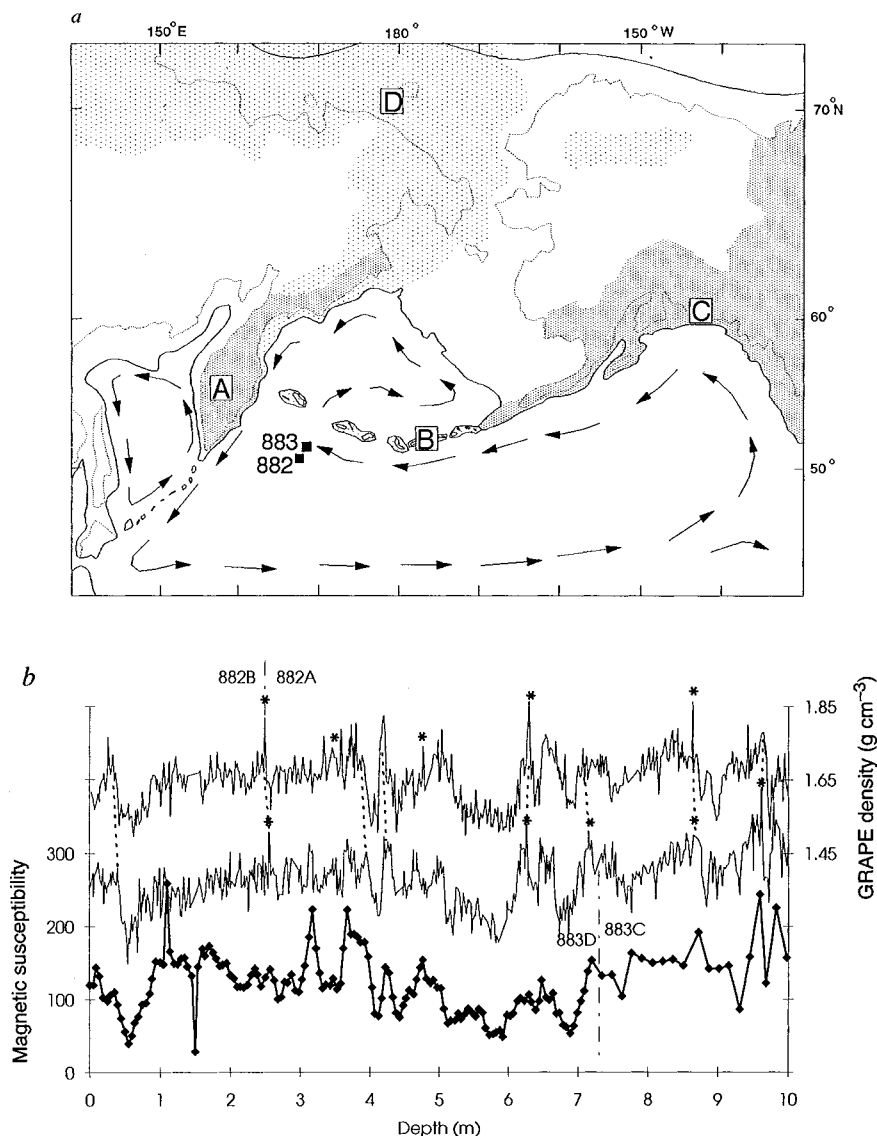
North Pacific the contrast in grain density and porosity between biogenic opal and terrigenous material creates large differences in GRAPE density. This is shown in Fig. 2a–d, where high biogenic opal content is associated with low GRAPE density (high porosity), whereas high terrigenous material content corresponds to high GRAPE density (low porosity). The main source of terrigenous material in these sediments is ice rafting. We therefore use variations in GRAPE density to infer changes in ratio of terrigenous material to biogenic opal. The good correlation between the GRAPE record and the lower-resolution magnetic susceptibility record (Fig. 1b), together with results from coarse-fraction analysis (Fig. 2c, d) support the association of high GRAPE densities and abundant IRD (occasional volcanic ash layers also have a high GRAPE density (Fig. 2c) and magnetic susceptibility). The main source of IRD in the Detroit Seamount area is generally thought to be the Okhotsk–Kamchatka region of Siberia^{8,12,14,22}. But the prevailing surface currents would not carry icebergs from this area, so one should also consider the Aleutian Islands^{8,9,23}, southern Alaska^{9,13} and ice sheets north of the Bering Straits²³ as possible sources (Fig. 1a).

The age model for Site 883 is based on oxygen isotope stratigraphy²⁴. This chronology is supported by a stratigraphy based on abundance of the radiolarian *Cycladophora davisiana* and the last occurrence of the radiolarian *Lychnocanoma nipponica sakai* (0.05 Myr ago)²⁵. In addition, two accelerator mass

spectrometry ¹⁴C (AMS) dates²⁵ for the upper part of hole 883D provide good chronological control. Because of the low abundance or complete absence of foraminifera²⁴, reflected in low CaCO₃ contents (Fig. 2d), a high-resolution $\delta^{18}\text{O}$ chronostratigraphy for Site 883 was not feasible. Nevertheless, the principal isotope stages are recognizable²⁴ in the $\delta^{18}\text{O}$ record (Fig. 3). The *Uvigerina* $\delta^{18}\text{O}$ record from Site 883 is shown with the SPECMAP timescale²⁶. The age model for Site 882 was constructed by correlating it to Site 883 by using the GRAPE records. The two GRAPE records are readily correlated visually (Fig. 1b). In addition, a few ash layers are present in both records and were used as tie points.

With the chronostratigraphy described above, GRAPE records from both sites were compared over the past 95 kyr with the $\delta^{18}\text{O}$ record from GRIP¹, Summit (Fig. 4, where the data are slightly smoothed). There is a close similarity between the ice-core $\delta^{18}\text{O}$ record and North Pacific GRAPE record; higher GRAPE values can be correlated with lower $\delta^{18}\text{O}$ values in GRIP. All major 'spikes' seen in the GRIP record can be tentatively identified in the GRAPE density record. Aware of the absence of a high-resolution $\delta^{18}\text{O}$ chronostratigraphy, we suggest that during times of low atmospheric temperature (identified from the GRIP record), there was increased discharge of icebergs into the North Pacific. Records from the North Atlantic show that these cooling cycles have culminated in enormous discharges

FIG. 1 a, Physiography and generalized surface circulation (indicated by arrows) of the northern North Pacific and marginal seas. The broken line indicates the modern continental outline. Modern 100-m isobath (approximate glacial shoreline) shown by solid line. Areas of extensive glaciations (indicated by stippled areas) have been adapted from refs 29 and 30. A (Kamchatka), B (Aleutians), C (southern Alaska) and D (Beringia) indicate possible source areas of IRD in the Detroit Seamount area. b, Correlation between Sites 882 and 883 (dotted line) for the upper 10 m, based on the GRAPE density record (thin lines), and the magnetic susceptibility record (filled diamonds and thick line) for Site 883. Dot-dashed lines indicate composite depth switch points between holes 882B and 882A, and between holes 883D and 883C, respectively. Ash layers are indicated by asterisks.



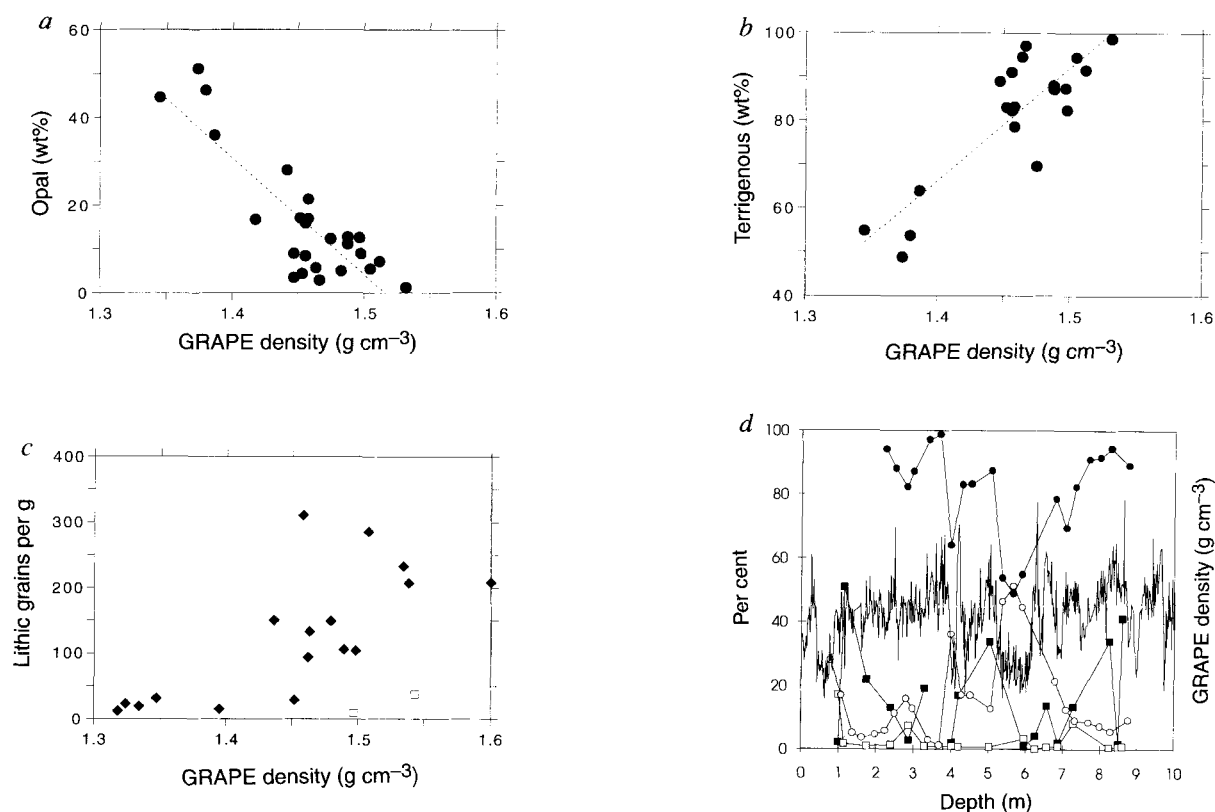


FIG. 2 *a*, Opal proportion (data from Ref. 31) plotted against GRAPE density for Site 882. The dotted regression line is plotted from $y = 402.18 - 265.26x$, $R = 0.84757$. *b*, Terrigenous material proportion plotted against GRAPE density for Site 882. The dotted regression line is plotted from $y = -294.14 + 257.43x$, $R = 0.85749$. *c*, Lithic concentration in numbers of grains $>63 \mu\text{m}$ per gram of total sediment (filled diamonds) plotted against GRAPE density for Site 882. Open squares

indicate samples where volcanic glass content is much higher than IRD content. *d*, Depth plotted against IRD ($>63 \mu\text{m}$) grain percentage (filled squares), CaCO₃ weight percentage (open squares), opal weight percentage (open circles, opal data from ref. 31), terrigenous material weight percentage (filled circles) and GRAPE density (solid line) for Site 882.

of icebergs into the North Atlantic (Heinrich events^{27,28}). Recently, Bond and Lotti⁵ have found that the amount of glacial ice discharges into the North Atlantic increased suddenly every 2,000 to 3,000 years, coincident with the Dansgaard-Oeschger cooling cycles¹, much more frequently than the 7,000–10,000-year pacing of enormous discharges of icebergs associated with Heinrich events.

Our data demonstrate that during the many brief cold events of the last glacial (Stages 2, 3 and 4), there were pulses of ice

rafted detritus input into the North Pacific Ocean, just as has been reported for the North Atlantic⁵. This important conclusion is valid whether or not the exact details of our correlation are in every case correct, and suggests that high-frequency climatic variability was probably a characteristic of the whole of the north high latitudes, rather than being a purely North Atlantic phenomenon. This in turn indicates that it is very important to discover the cause of this variability. It is crucial to verify the source of the IRD; the IRD flux that we observe is broadly

FIG. 3 Oxygen isotope results of *Uvigerina* sp. from Site 833 (filled circles, data from ref. 24) and GRAPE density for the upper 10 m. Oxygen-isotope stage boundaries are shown by dotted lines. Solid arrows indicate AMS datings for hole 883D (13.63 and 20.33 kyr, data from ref. 25). The last occurrence (LO) of radiolarian *L. nipponica sakai* (50 kyr) is also shown (data from ref. 25).

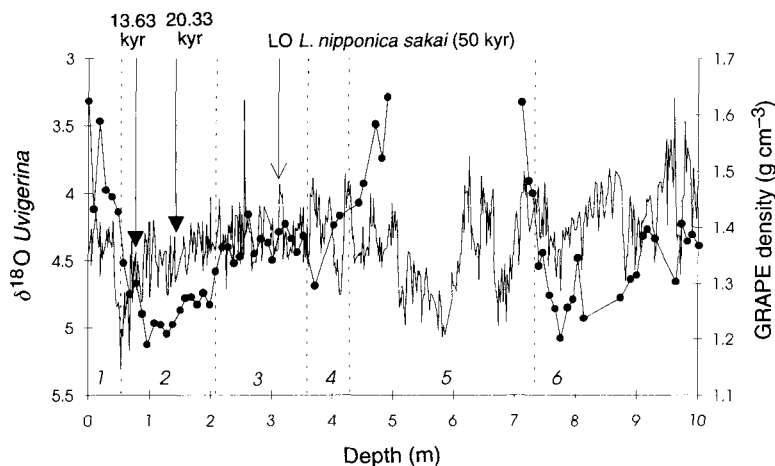
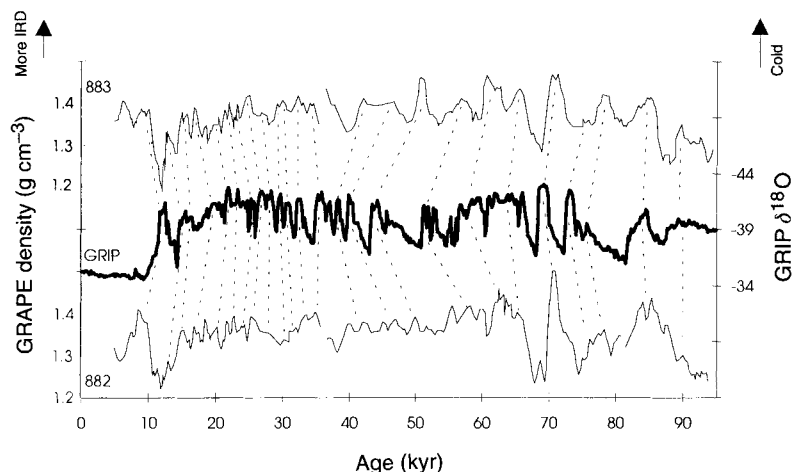


FIG. 4 Age plotted against GRAPE density records from ODP Sites 882 and 883 (thin lines), and oxygen isotope record from the GRIP ice core in Summit, Greenland¹ (thick line). Possible correlation between GRAPE records and the GRIP oxygen isotope record is shown by dotted lines.



similar to that observed in North Atlantic cores. If the IRD originated in a Siberian ice sheet, a possible mechanism linking North Pacific and North Atlantic events could be sea level. If, in contrast, the chief source is glaciers calving icebergs into the ocean, the relationship that we observe could be attributed to major fluctuations in air temperature that controlled the area of glaciers entering the North Pacific, and the air temperature over Greenland. In that case (which we consider more likely), unravelling the cause of such large temperature fluctuations affecting such a large part of the Northern Hemisphere should be an important goal for future research. □

Nitrogen content of the mantle inferred from N₂-Ar correlation in oceanic basalts

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RARE gases have proved to be particularly useful in modelling the early evolution of the Earth's atmosphere¹⁻³. But it is not straightforward to extend this approach to the main volatile species (such as hydrogen, carbon and nitrogen) that comprise the atmosphere, hydrosphere and sediments, as these elements are chemically reactive and may have experienced different geodynamic histories. A way around this problem is to calibrate major volatile species relative to rare gases⁴⁻⁸. Here I use a recently developed static mass spectrometry method that allows simultaneous analysis of nitrogen, carbon, helium and argon⁹ to analyse gases trapped in vesicles of mid-ocean-ridge basalt glasses. The results show that the abundances of N₂ and ⁴⁰Ar (a radiogenic isotope that has been produced through geological time by the decay of ⁴⁰K in the solid Earth) correlate well over several orders of magnitude, suggesting that the N₂/⁴⁰Ar ratio in the mantle source is near-constant and comparable to the present-day atmospheric value. In contrast, the inferred mantle N₂/³⁶Ar ratio (where ³⁶Ar is a primordial isotope of argon) is two orders of magnitude higher than the atmospheric ratio. This observation, when combined with argon isotope systematics, allows a better estimate to be made of the nitrogen content of the mantle.

The geological history of nitrogen is not well understood. The study of this element in mantle-derived samples is severely complicated by its generally very low abundance⁹⁻¹², which makes nitrogen sensitive to atmospheric contamination and, more generally, to addition of surface-derived nitrogen (such as organic N). Calibration of nitrogen relative to argon in mantle-derived samples presents several advantages. First, it provides the opportunity to evaluate surface contamination because the ⁴⁰Ar/³⁶Ar ratio in the mantle is much higher than the atmospheric value of 295.5. Second, ⁴⁰Ar originates from the radiogenic decay of ⁴⁰K, providing a well constrained chronological and geochemical framework, whereas ³⁶Ar (or ³⁸Ar) is primordial in origin.

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TABLE 1 MORB crushing data

Sample	Type	Vesicularity (%)	^{40}Ar (10^{-12} mol g $^{-1}$)	N_2 (10^{-10} mol g $^{-1}$)	$^{40}\text{Ar}/^{36}\text{Ar}$	$\pm$	$\text{N}_2/^{40}\text{Ar}$	C/N	$^4\text{He}/^{40}\text{Ar}^*$	(C/N) $_0$
EPR 21° N										
981R26	N	0.18	5.68	4.82	3,321	167	85	—	13	—
EPR 13° N										
CY82 09 03	N	0.65	1.35	0.94	3,020	7	70	1,561	110	292
CY82 31 02	N	0.53	1.45	1.09	1,065	3	75	1,032	44	296
CL DR01 5V	N	1.25	24.0	32.4	4,043	9	135	368	15	176
CY82 30-b	E	0.40	2.38	1.61	1,668	4	68	676	42	199
MAR 36° N										
CH31DR09	T	0.50	12.8	6.25	1,200	2	49	2,088	7.8	1,335
CH31 DR11	E	4.15	32.3	17	10,828	485	53	4,606	13	2,358
CH31 DR10	E	0.81	1.21	0.96	532	1	79	2,109	472	201
MAR 30° N										
CH98 DR11	N	0.19	20.7	17	1,273	25	82	418	6.2	298
CH98 DR12	N	0.40	18.6	19.4	9,717	602	104	369	6.6	256
CH98 DR15	N	0.33	106	118	8,756	24	111	193	4.5	160
CH98 DR17	N	0.86	77	53.5	2,279	4	70	408	7.7	263
MAR 14° N										
21D43	E	16.8	803	445	1,641	2	55	215	1.17	
Air					295.5		83.6			

All samples are fresh MORB glasses of the normal (N), transitional (T) and enriched (E) types from the East Pacific Rise (EPR) and the Mid-Atlantic Ridge (MAR). Gases were extracted after on-line vacuum crushing⁹. Sample vesicularity was determined by point counting or image processing. Typical blanks were 1.4×10^{-11} mol N_2 and 10^{-14} mol ^{40}Ar . Uncertainties on abundances are estimated to be 10% for Ar and 20% for N_2 (2σ). 21D43 is a split of a popping rock analysed for rare gases by Staudacher *et al.*²⁰ who found $^{40}\text{Ar}/^{36}\text{Ar}$ ratios varying from 560 to 28,000, and for major gases by Javoy and Pineau¹². He and CO_2 abundances will be discussed in detail elsewhere. $^{40}\text{Ar}^*$ refers to the amount of mantle-derived ^{40}Ar computed assuming that all ^{36}Ar is atmosphere-derived, which is valid at a first approximation considering the extremely small amount of ^{36}Ar in the MORB mantle. (C/N) $_0$ is the carbon/nitrogen ratio corrected for melt-vapour fractionation (see text). Sample 21D43 is not corrected for fractionation because its $^4\text{He}/^{40}\text{Ar}^*$ ratio is lower than the inferred initial value of 3 ± 1 .

We have developed a new static mass spectrometry method that allows the simultaneous analysis of major volatiles and rare gases, including the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio⁹. After extraction following either stepwise heating or vacuum crushing, gases were separated into several fractions (He, Ar and N_2), purified and analysed sequentially for He, N_2 and Ar contents as well as He and Ar isotopic ratios, while the total gas pressure was measured with a capacitance manometer for CO_2 content. Analysis of the total amounts of gases extracted from MORB glasses shows that the bulk nitrogen content is variable and does not correlate with either major or trace elements nor rare gases (B. M., manuscript in preparation). In particular the $\text{N}_2/^{40}\text{Ar}$ ratio varies between 192 and 29,600 irrespective of the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio. These observations suggest that part of the total nitrogen is of secondary derivation (for example, organic N or N-rich alteration microphase), a view consistent with the somewhat erratic nitrogen isotopic ratios observed in MORB^{10,11}. In contrast, the analysis of volatiles extracted after vacuum crushing of MORB glasses shows a striking uniformity of the $\text{N}_2/^{40}\text{Ar}$ ratio, although vesicularity and total gas content vary over several orders of magnitude (Table 1). The MORB mean $\text{N}_2/^{40}\text{Ar}$ ratio is 80 (± 25) for 13 fresh MORB glasses from various locations, in general agreement with a previous measurement of 50 for a single gas-rich MORB sample¹² and comparable to the $\text{N}_2/^{40}\text{Ar}$ ratio in air of 83.6. It must be emphasized that this similarity is not the result of air (or seawater) contamination because in all cases the associated $^{40}\text{Ar}/^{36}\text{Ar}$ ratios are different from the atmospheric value (Table 1). It is also important to note that the near-constancy of the $\text{N}_2/^{40}\text{Ar}$ ratio, despite extreme variations of the total gas content (as represented by vesicularity) and of the radiogenic $^4\text{He}/^{40}\text{Ar}^*$ ratio (an index of volatile fractionation; see below), shows that N_2 does not fractionate extensively with respect to Ar during magma degassing (Fig. 1). These results confirm an independent suggestion that N_2 and Ar solubilities in basaltic melt seem comparable⁹. As Ar is incompatible during partial melting (see, for example, ref. 13), the constancy of the N_2/Ar ratio suggests that this is also the case for nitrogen. The mean $\text{N}_2/^{40}\text{Ar}$ ratio of 80 is therefore regarded as representative of the $\text{N}_2/^{40}\text{Ar}$ ratio in the MORB mantle. The present-day flux of N_2 from the mantle, computed relative to that of He (8.3×10^7 mol yr^{-1} ; ref. 14) and from the mantle $^4\text{He}/^{40}\text{Ar}^*$

radiogenic production/accumulation ratio (3 ± 1 ; ref. 1), is estimated to be $2.2 \pm 1.0 \times 10^9$ mol N_2 yr^{-1} and the bulk terrestrial abundance of nitrogen, assuming a K abundance of 300 ± 60 p.p.m. for the silicate Earth, is $2.8^{+1.0}_{-0.8}$ p.p.m.

The present data also permit a calculation of the C/N ratio of the mantle. A mean C/N ratio of 395 ± 30 was previously reported for a gas-rich MORB sample¹². The C/N ratios of the present samples (Table 1) show a large scatter between 215 and 4,606 with a mean of $\sim 1,200$. But these ratios need to be corrected for melt-vapour fractionation because the solubilities of CO_2 and Ar (and therefore N_2) are markedly different. The radiogenic $^4\text{He}/^{40}\text{Ar}^*$ ratio can be used for correcting such fractionation (Fig. 1) because (1) the source ratio can be reasonably assumed to be that of the radiogenic production/accumulation in the mantle and (2) He and Ar solubilities differ by a factor of 10, resulting in large $^4\text{He}/^{40}\text{Ar}^*$ variations in MORB glasses (see, for example, ref. 1). Whereas the N_2/Ar ratios remain constant, independent of the $^4\text{He}/^{40}\text{Ar}^*$ variations (thus confirming that the N_2 and Ar solubilities are similar), the C/N ratios increase strongly with increasing $^4\text{He}/^{40}\text{Ar}^*$ ratios (Fig. 1). Closed-system fractionation would predict a maximum $^4\text{He}/^{40}\text{Ar}^*$ ratio equal to the radiogenic ratio multiplied by the ratio of Ar/He solubilities, that is, ~ 30 (see, for example, ref. 13). However, several of the samples show higher values, suggesting that open-system fractionation took place.

Thus I assume a Raleigh distillation process which, for two volatile species x and y , can be written:

$$(x/y)_r = (x/y)_o f_y^{[(K_x/K_y) - 1]}$$

where suffixes o and r refer respectively to initial and residual ratios, f_y is the fraction of volatile y remaining in the liquid and K_i are the solubility (Henry) coefficients. In the case of four species (He, C, N and Ar), the equation set can be resolved for f_i and the source C/N ratio can be computed according to:

$$(C/N)_o = (C/N)_r \left[\frac{(^4\text{He}/^{40}\text{Ar}^*)_r}{(^4\text{He}/^{40}\text{Ar}^*)_o} \right]^{[\frac{K_{\text{N}_2} - K_{\text{CO}_2}}{K_{\text{He}} - K_{\text{Ar}}}]}$$

with K_{He} , K_{CO_2} and K_{Ar} equal to 2.5×10^{-11} mol g $^{-1}$ hPa $^{-1}$ (ref. 15), 9.0×10^{-12} mol g $^{-1}$ hPa $^{-1}$ (ref. 16) and 2.6×10^{-12} mol g $^{-1}$ hPa $^{-1}$ (ref. 15), respectively (for nitrogen, of which the solubility

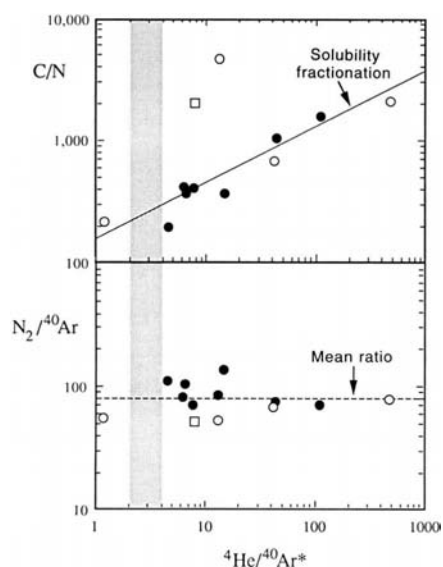


FIG. 1 $N_2/^{40}\text{Ar}$ and C/N ratios as a function of the radiogenic $^4\text{He}/^{40}\text{Ar}^*$ ratio. For the former no attempt was made to extract radiogenic $^{40}\text{Ar}^*$ from the data as the $N_2/^{40}\text{Ar}$ ratios of the mantle and of the atmosphere are identical within analytical uncertainties. Filled circles, N-MORB; open circles, E-MORB; squares, T-MORB. The solid line in the upper part of the graph represents solubility fractionation computed with the equation given in the text. The initial C/N ratio has been taken as 247, the mean corrected C/N value. Taking other initial values would result in lines having the same slope. The shaded area represents the range of the radiogenic $^4\text{He}/^{40}\text{Ar}^*$ ratio for production/accumulation in the mantle.

is not precisely known, a value of $3.7 \times 10^{-12} \text{ mol g}^{-1} \text{ hPa}^{-1}$ is assumed⁹). Using these values, the corrected (C/N)_o ratio (Table 1) is found to have a mean of ~500.

Two samples deviate significantly from the correlation in Fig. 1, suggesting a source effect in addition to volatile fractionation. It is interesting to note that these two samples are from the FAMOUS area (Mid-Atlantic Ridge at 36° N) near the Azores platform where the C/³He ratios higher than typical MORB values and correlating with Sr isotopic ratios have been interpreted as reflecting the addition of a subducted, carbon-rich component¹⁷. Excluding the FAMOUS data yields a minimum C/N ratio of 238 ± 54 , possibly representative of the depleted mantle; the minimum C/N ratio of the bulk silicate Earth (surface + mantle) is 134 ± 48 . No extraterrestrial sources could supply volatiles with such a C/N ratio because all of them (for example, meteorites and solar wind) exhibit much lower C/N values in the range 4–30 (ref. 18). Exceptions are C3 chondrites, which show C/N ratios in the range 90–130, but their oxygen isotope composition prohibits any genetic relationship with Earth's building material (see, for example, ref. 19). The C/N ratio of the silicate Earth is thus a characteristic feature of our planet.

In contrast with the constant $N_2/^{40}\text{Ar}$ ratio, the $N_2/^{36}\text{Ar}$ ratio varies over two orders of magnitude (Fig. 2). Because variations in the $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in mantle-derived samples are unambiguously attributed to variable contamination of a pure mantle end-member ($^{40}\text{Ar}/^{36}\text{Ar}$ up to 28,000; ref. 20) by atmospheric argon (see, for example, ref. 1), variations in the $N_2/^{36}\text{Ar}$ ratio may also result from two-end-member mixing between an atmospheric pole ($N_2/^{36}\text{Ar} = 2.47 \times 10^4$ in air; $\sim 1.2 \times 10^4$ in air-saturated water) and a mantle end-member having much higher $N_2/^{36}\text{Ar}$ and $^{40}\text{Ar}/^{36}\text{Ar}$ ratios. Taking a $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of 28,000 for the mantle end-member and a $N_2/^{40}\text{Ar}$ ratio of 80 allows a mantle $N_2/^{36}\text{Ar}$ ratio of 2.2×10^6 to be calculated. In the $N_2/^{36}\text{Ar}$ – $^{40}\text{Ar}/^{36}\text{Ar}$ diagram (Fig. 2) the points fit the curves representing mixing between atmospheric and mantle end-mem-

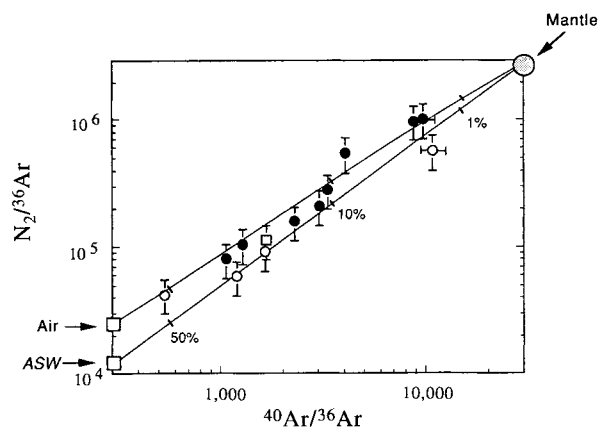


FIG. 2 $N_2/^{36}\text{Ar}$ ratio as a function of $^{40}\text{Ar}/^{36}\text{Ar}$ ratio. Same symbols as in Fig. 1. The solid lines represent mixing curves between the atmosphere as represented by air or air-saturated water (ASW), and a mantle pole having $N_2/^{36}\text{Ar}$ and $^{40}\text{Ar}/^{36}\text{Ar}$ ratios of 2.2×10^6 and 28,000, respectively. The fraction of atmospheric N_2 in the mixture is represented on the curves as a percentage of total N_2 .

bers, confirming the proposed mixing hypothesis. Thus, while the $N_2/^{40}\text{Ar}$ (or the time-integrated nitrogen/potassium) ratio of the mantle is similar to the $N_2/^{40}\text{Ar}$ ratio at the surface of the Earth, the surface reservoir is enriched in ^{36}Ar relative to nitrogen by two orders of magnitude. Considering nitrogen trapped in sedimentary or crystalline rocks would not change this conclusion because its abundance ($0.44 \times 10^{20} \text{ mol}$; refs 4, 21) is lower than that of atmospheric nitrogen ($1.36 \times 10^{20} \text{ mol}$).

Based on the analysis of the vesicle fraction of a gas-rich MORB glass by Javoy and Pineau¹², Zhang and Zindler³ recognized this difference and proposed that recycling of even minute amounts of surface-derived nitrogen to a drastically volatile-depleted mantle region might have increased the mantle ratio by two orders of magnitude. This view is compatible with elemental abundances of volatiles but may need revision from the isotope point of view because the isotopic composition of nitrogen in the above-mentioned gas-rich MORB ($\delta^{15}\text{N} = -5\%$; ref. 12) is clearly different from that of sedimentary nitrogen involved in subduction processes ($\delta^{15}\text{N} \sim +5\%$; refs 22, 23). ($\delta^{15}\text{N}$ (in units of per mil) is defined as $\{[(^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{air}}] - 1\} \times 1,000$.) Ancient subduction could be an alternative because negative $\delta^{15}\text{N}$ values down to $\sim 6\%$ have been reported for 3.5-Gyr organic matter, whereas younger organic matter (for example 2.0 Gyr) presents a $\delta^{15}\text{N}$ distribution similar to the present-day one²⁴. But it is not clear whether these negative values reflect the isotopic composition of ancient atmospheric nitrogen or metabolic or diagenetic effects.

Alternatively the two orders of magnitude difference in the $N_2/^{36}\text{Ar}$ ratio between the surface and the interior of the Earth may well be due to early depletion of nitrogen relative to ^{36}Ar in a primitive atmosphere. In this instance, the similar mantle and atmosphere $N_2/^{40}\text{Ar}$ ratios would not be mere coincidence, but rather the result of parallel degassing of N_2 and ^{40}Ar over the history of the Earth. This is consistent with the distribution of N_2 between the mantle and the Earth's surface: 49% (+19/–14%) is presently at the surface, a similar proportion to that of radiogenic Ar ($41 \pm 10/–7\%$ at the surface). A better assessment of the isotopic signature of nitrogen in the mantle should place firm constraints on the proportion of each contribution (primitive atmosphere versus mantle degassing), and, more generally, on the processes that have contributed nitrogen to the terrestrial atmosphere. □

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Coprolites as evidence for plant–animal interaction in Siluro–Devonian terrestrial ecosystems

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A FEW remarkable finds document the colonization of land by animals and plants in the mid-Palaeozoic^{1–3}, but much rarer is unequivocal evidence for plant–animal interaction^{4,5}. Here we announce the discovery of coprolites (fossil faeces) in Upper Silurian (412 Myr) and Lower Devonian (390 Myr) rocks from the Welsh Borderland that pre-date examples of similar composition in the Carboniferous by about 90 million years^{6,7}. The majority consist predominantly of undigested land-plant spores with varying proportions of cuticles, tubes and less readily identifiable (presumably plant) material. Because coeval animal fossils of suitable size are carnivores⁸, direct evidence for the coprolite producers is lacking, but we speculate that they could have been spore eaters (and hence the earliest example of herbivory of higher plants) or detritivores similar to modern millipedes. In either case, they demonstrate the cycling of primary productivity in early terrestrial ecosystems.

The majority of coprolites were recovered by hydrofluoric acid bulk maceration of fluvial siltstones (overbank flood deposit) from a Lower Devonian stream-side locality (Mid Lochkovian: mid *micromnatus*–*newportensis* Spore Biozone) on North Brown Clee Hill, renowned for its excellent cellular preservation in plant mesofossils⁹. The three Silurian examples occurred in marginal marine facies (Ludford Lane (1) and Perton Lane (2)), of Přídolí age. Ludford Lane has yielded the earliest terrestrial animals¹

and Perton Lane has a diverse rhyniophytoid assemblage¹⁰. At all localities, macerates also include axial, commonly fertile, higher plant fossils, and cuticles of higher plants, '*Nematolithus*', eurypterids and 'myriapods'.

In that they contain spores, the coprolites (Fig. 1) were initially thought to be isolated sporangia, but their regular shape, lack of an enclosing sporangial wall, presence of more than one spore type (up to nine in some specimens: 25 different spore types in total; Table 1), varying proportions of cuticles, tubes and less readily identifiable plant debris, rule out this possibility. As sedimentological evidence shows that the coprolites have drifted from near or far into these marine and fluvial depositional environments, were they produced by terrestrial or aquatic animals? Evidence favouring aquatic ones is the presence of cuticles of possible aquatic animals (eurypterids, scorpions and kampecarid 'myriapods') in the same sequence, though some or all of these may have been terrestrial or amphibious in the Devonian^{11–13}. Evidence for a land origin is considered more compelling in that the coprolites are consistently associated in both marine and freshwater facies with predominantly land-derived debris of the same preservation characteristics.

Were the coprolite producers feeding on living plants (herbivores) or on dead plant material (detritivores)? Considering the first possibility, extant herbivores exploit gut fungi and bacteria to degrade cellulose, but even with the assistance of such 'biochemical brokers'¹⁴, the nutrient value of living vegetative tissues would have been low. Sporangia and spores would have provided an energy-rich diet of presumably relatively high nitrogen content^{15,16}, and thus spore feeding becomes an attractive possibility. Indeed, it has already been suggested that pollen feeding preceded herbivory in insect evolution^{7,14}. This would explain why large numbers of a single spore type dominate many coprolites, whereas the higher frequency of tetrads (in comparison with dispersed spore assemblages) suggests that immature sporangia were eaten. It would also account for the presence of spines on sporangia¹⁰. The question then arises as to whether spores and pollen are directly comparable in availability of spore contents because sporopollenin itself is unlikely to have been digested. Extant pollen feeders obtain such nutrients by either mechanical lysis (for example, maceration in beetles) or chemical means (such as secretion of enzymes and/or simple diffusion in flies¹⁷). The first seems unlikely for our animal as so few spores are incomplete or broken and often remain in tetrads. Those that are cracked open are larger, usually smooth, ones (Fig. 1j), suggesting accidental breakage on passage through the animal or during post-depositional compression rather than deliberate lysis. Considering diffusion, our ultrastructural studies show¹⁸ that the fossil spores lack any areas of wall without sporopollenin (compare colpi and sulci of pollen) and that in *in situ* spores the trilete mark consists of a fold in the exospore, a slit presumably developing before germination. Diffusion would have had to occur through microcapillaries in the sporopollenin-impregnated wall. The limited available experimental data (on green mature *Onoclea* spores¹⁹) indicate that only very small molecules (smaller than glycerol) can pass through, and not macromolecules such as enzymes, although permeability increases with age of mature spore and proximity to germination. Thus, extraction of nutrients by diffusion alone seems unlikely, and diet might have been supplemented with extra-exospore material such as perispore or locular fluids. However, as sporangial walls were composed of thick-walled cells and were highly cuticularized, it is puzzling that they are not found more commonly, in coprolites (Fig. 1q) if the animals were spore feeders.

Another explanation is that the animals were feeding on litter rich in spores and spore masses. The former would explain the diversity in the spores found in very small numbers, and the latter might reflect dispersal in clumps in certain taxa (for example, *Cooksonia pertoni*⁹) or production of sporangia at ground level (for example, certain extant hepatics, where spores also sometime occur in tetrads). The high spore concentration

FIG. 1 All examples Devonian (HD) except *a* and *l* (Přídolí: LL and PL). *a*–*e*, Range in shape of more regular and complete coprolites (40% of assemblage): 3.3×1.27 to 0.53×0.22 mm. *a*, Elliptical, with mainly cuticle and occasional spores (LL1 $\times 87$). *b*, Elongate with slight asymmetry, mainly spores (HD1 $\times 17$). *c*–*d*, Obliquely truncated (compare with modern millipedes), outline varying with degree of compression and composition. Spore-dominated forms have smooth outlines except when impregnated by pyrite, when they are three-dimensional and knobbly. (*d*, HD4 $\times 24$; *p*, HD16 $\times 185$). Cuticle- and unidentifiable-plant-debris-dominated forms are usually less regular, with numerous voids separated by draped debris (*c*, HD3 $\times 18$; *e*, HD5 $\times 32$). Many are partly compacted and show broad, ill-defined, oblique, shallow depressions (*d*, *e*). *e*, Contains mangled tissue from new tetrad-containing plant. *f*, *Laevolancis divellomedia* (of dyad origin), with amorphous slime (possibly anal-gland glue) and some cuticle (HD6 $\times 190$). *g*, *Pero-trilites* sp. with wrinkled outer layer and adhering apiculate forms (HD7 $\times 490$). *h*, Apiculate monads including *Streelispora newportensis*, *Aneurospora* sp. and ?*Emphanisporites* sp. (HD8 $\times 668$). *i*, Undescribed spore of dyad origin (HD9 $\times 615$). *j*, Large laevigate, split disk, intact *Streelispora newportensis* (papillate) and example of rare piece of indeterminate, probably higher plant, tissue (sterome?) (HD10 $\times 500$). *k*, Undescribed apiculate spore (dyad origin) with small apiculate tetrad (HD11 $\times 780$). *l*, Extremely well-preserved *Apiculiretusispora*; cf. *synorea* (PL1 $\times 1,660$). *m*, Possibly *Retusotriletes warringtonii* (HD13 $\times 950$). *n*, *Archaeozonotriletes* sp. with plant debris (HD14 $\times 455$). *o*, Indeterminate laevigate spores with interspersed cuticular sheets; latter are commonly stacked, fibrillar but rarely powdery; stomata have not been seen (HD15 $\times 235$). *p*, Laevigate tetrads filled with pyrite (HD16 $\times 190$). *q*, Fragments of sporangial cuticle (HD17 $\times 190$). *r*, Unique example of lumen cast of tracheid, grooves marking positions of spiral secondary thickening (HD18 $\times 1,880$).

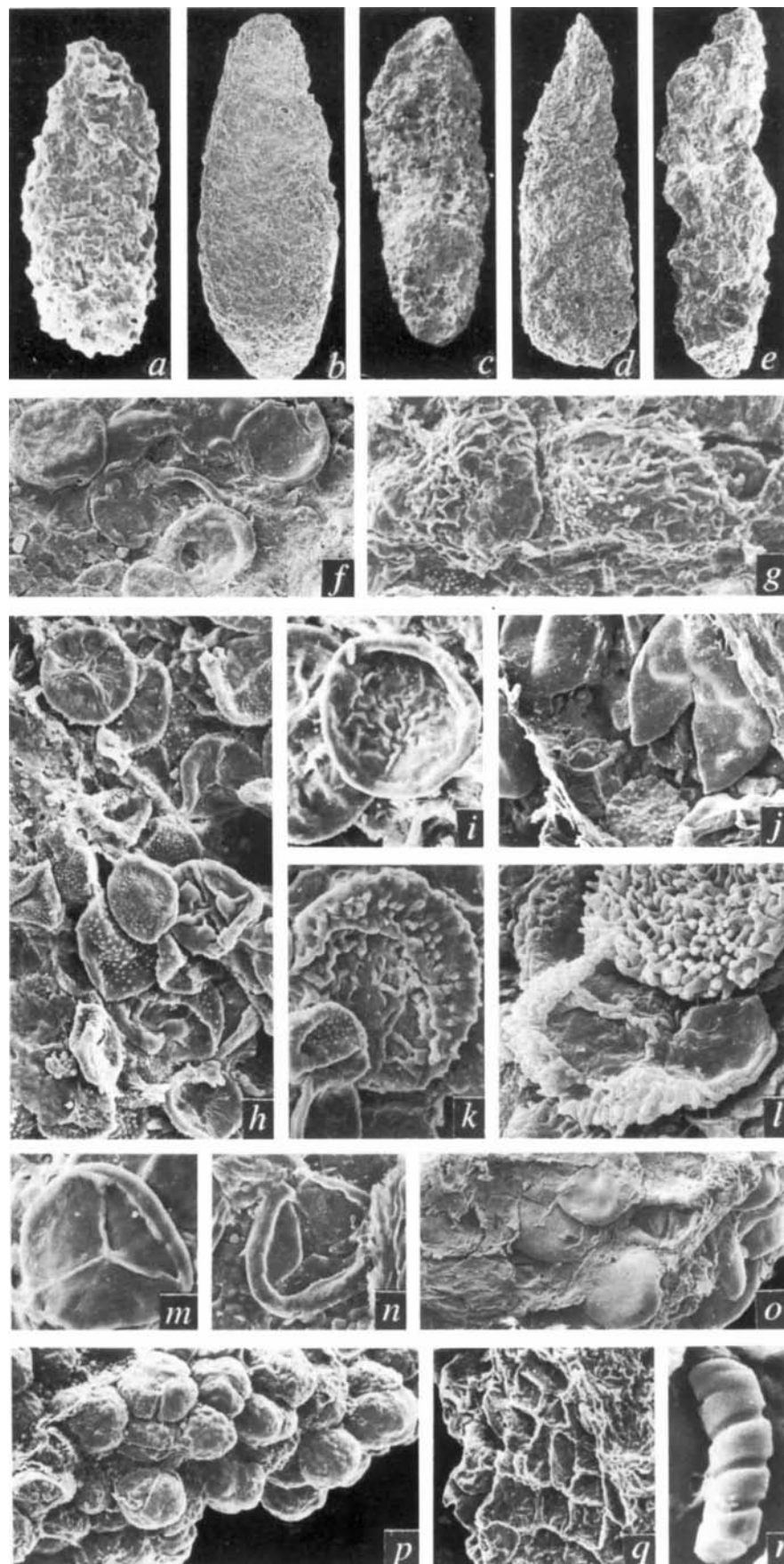


TABLE 1 Dispersed spore taxa recorded in coprolites and possible parent plants

Spore taxon	Frequency	Occurrence	Source	Fig.
<i>Ambitisporites avitus</i>	r	mt		1o
<i>Ambitisporites dilutus</i>	c	m	? <i>Cooksonia pertoni</i>	1m
<i>Ambitisporites</i> sp. (S)	r	m	? <i>Cooksonia pertoni</i>	
<i>Aneurospora</i> sp. (many varieties)		m	<i>Cooksonia pertoni</i> / <i>Salopella</i> sp.	1h
Apiculate cryptospores (4 new species)	c	d		1k
Apiculate/granulate tetrad	vr			1k
<i>Apiculiretusispora</i> cf. <i>synorea</i> (S)	c	mt	P (discoidal)	1l
<i>Archaeozonotriletes dubius</i>	r	mt		1n
<i>Artemopyra</i> sp. (cryptospore)	r			
<i>Chelinospora</i> sp.	vr	m	P	
<i>Cymbotrilites</i> sp.	r	t		
<i>Emphanisporites microrhatus</i> ?	r	m		
<i>Emphanisporites</i> sp.	r	m	P	
<i>Hispanaedisca</i>	r?	d		
Laevigate tetrads, large	c			1j
Laevigate tetrads, small	r			
Laevigate tetrads with low ornament	c			1p
<i>Laevolancis divellomedia</i>	r	d	P	1f
<i>Perotrilites</i> sp.	r	t	P	1g
? <i>Retusotrilites warringtonii</i> n. sp?	vr	m	'Zosterophylls'	1m
<i>Streelispore newportensis</i>	c		<i>Cooksonia pertoni</i>	1h,j
<i>Synorisporites verrucatus</i> (S)	c	m	P (discoidal)	
<i>Synorisporites</i> n. sp.	r	mt		

All Lower Devonian, except (S), which indicates Silurian. c, Common; r, rare; vr, very rare; m, monad; t, tetrad origin; d, dyad origin; P, present in as-yet unnamed sporangia. Not all spores can be matched with published dispersed spore taxa. This particularly applies to some of the commonest spores (apiculate monads in Fig. 1h) and laevigate tetrads (Fig. 1p), where details of distal and proximal surfaces are needed for identification. Difficulties also arise in identifying contorted or partially covered spores and those draped with a featureless film quite distinct from macerated cuticle (Fig. 1f).

in coprolites could result from digestive processes, coprophagy, or selective feeding. Litter feeders eat a variety of food: softer (for example, parenchymatous) tissues are more readily digested or compacted, and less recognizable in faeces. This might also account for the lack of identifiable fungal remains in the coprolites. Bacteria and fungi are important components of litter as both food sources and as endosymbionts for detritivores. Bacterium-sized particles in certain coprolites may be Recent, although precautions were taken to avoid contamination. Many litter arthropods ingest faeces, further concentrating more resistant items such as spores or cuticles. Selective feeding is perhaps the least plausible explanation, although 'leaky' spores might provide an energy source, as would the remains of extra-exosomal material and any saprotrophs living on it. However, apart from a few examples with roughened surfaces, the spores show no signs of bacterial or fungal damage²⁰, and are notable for the clarity of their surface detail—points in favour of sporangial feeding. Finally, overrepresentation of spore-bearing coprolites might result from choosing specimens for scanning electron microscopy. Feeding experiments with millipedes show that faecal pellets dominated by non-spore plant material are less regular in shape and so their fossils could be overlooked.

Whereas evidence for the feeding habits of the coprolite producers is equivocal, that for the animals themselves is even more obscure. Size of the coprolites would exclude most collembolans, mites and nematodes (too small), and earthworms (too large). Comparison with animals that are known or are likely to have evolved by Devonian times suggests that arthropods are the best contenders and that, for detritivores, 'myriapods' and large collembolans are the front runners. Although rather small kampeparid 'myriapods' occur in the same beds as the coprolites, little is known of their biology¹³. The oldest diplopod occurs in the late Silurian of Scotland, but the habitat (aquatic rather than terrestrial) of this and later Lower Devonian millipedes remains contentious²¹. Fossils of unequivocal herbivores are lacking from early terrestrial ecosystems, but indirect evidence for their activity comes from stems with wound response tissues, possibly due to chewing, and stained lesions, possibly a response to sap sucking which would leave no solid faeces. The few examples of appropriately sized extant embryophyte-spore feeders (such as ants²²

and moth caterpillars (D. Carter, personal communication)) come from groups not likely to have evolved by Silurian times.

In the light of such uncertainties, discussion on the relevance of our findings to the evolution of terrestrial ecosystems is perhaps premature. If the coprolite producers were detritivores, they demonstrate a logical extension of the microphytophagy first evidenced in the Silurian by faecal pellets of ascomycete hyphae^{23,24} and show that vegetation was being recycled through decomposer levels, as in soil and litter communities today. If herbivores, they add weight to the hypothesis that feeding on sporangia may have been more common and pre-dated that on vegetative organs¹². Given the sparse evidence for plant damage in the Devonian and Carboniferous¹⁷ and vertebrate herbivores until the Permian²⁵, it is possible that widespread herbivory did not arise until latest Palaeozoic times. □

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Locomotor performance of insects with rudimentary wings

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THE evolution of flight in insects triggered an unparalleled radiation and diversification such that flying insects comprise approximately two-thirds of all species¹, yet a gap in the fossil record obscures the origins of wings and flight². Among modern insects, stoneflies are morphologically primitive for several flight-related traits, which makes their locomotor behaviour and physiology of particular interest³. Here we show that *Allocaenia vivipara* stoneflies use a non-flying form of aerodynamic locomotion which may exemplify a precursor to flight. They raise their wings in response to wind, thereby sailing across water surfaces, but they are incapable of flapping. Sailing performance improves steadily with increasing wing size, and even the smallest wings significantly increase sailing velocity compared to wingless individuals. Performance during aerial gliding is less affected by wing size, which suggests that sailing is a more plausible setting for wing evolution. These results support the hypothesis that insect wings evolved from articulated gill plates of aquatic ancestors through an intermediate semi-aquatic stage⁴.

Previous studies of insect flight evolution have assumed that aerial gliders preceded powered fliers^{2,3,5,6}. However, the preponderance of evidence indicating that wings evolved from articulated gill plates used by aquatic forms for ventilation and/or locomotion^{7,8} suggests that the potential of proto-wings to enhance semi-aquatic locomotion should be explored.

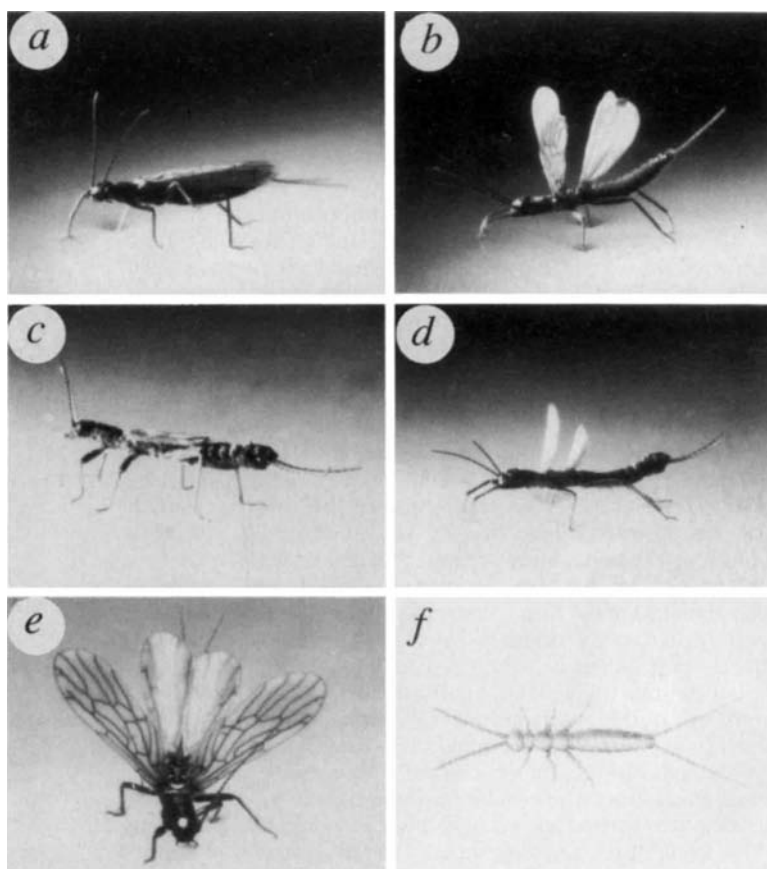
Accordingly, we recently studied a stonefly (*Taeniopteryx burksi*; Plecoptera: Taeniopterygidae) that uses wing-flapping locomotion to move on the water surface⁴. Surface-skimming is effective even when wing size is greatly reduced and muscle power output is minimized. Here we extend that study by examining aerodynamic performance of a non-flapping stonefly whose anatomy, physiology and behaviour may even more closely approximate that of the first winged insects.

In the northeastern United States, *Allocaenia vivipara* stoneflies (Plecoptera: Capniidae) emerge as adults from stream-dwelling aquatic nymphs during mid-January through to April. Females are uniformly long-winged (70–105% of body length), whereas males are mostly short-winged (33–65% of body length). Individuals that emerge as adults on midstream rocks, sticks or ice stand on top of the water surface tension and raise their wings in response to gusts of wind (Fig. 1), thereby sailing across the surface and reaching shore at locations where the wind is perpendicular to the direction of current flow. Even at warm laboratory temperatures, these stoneflies never flap their wings.

We built a wind tunnel over a water surface to examine the effect of wing size on sailing performance. At all wind speeds tested, sailing velocity improved linearly with increasing wing length (Fig. 2), and stoneflies with the smallest wings had significantly improved sailing performance compared to 'wingless' individuals (those that did not raise their wings). Relative wing length of *A. vivipara* males (wing length/body length) is as low as 0.33, a value similar to the size of articulated, moveable gill plates of early pterygote insect nymphs from the Carboniferous and Permian⁷. Thus gill plates of protopterygotes might have been useful as sails if these insects were active on the water surface, as many extant apterygote insects are⁹, and raised their gill plates in response to wind.

Allocaenia stoneflies also raise their wings and glide when they are dropped into air (a behaviour that appears to be used only rarely in nature), and thus it is possible to compare the func-

FIG. 1. Surface sailing in the laboratory in *A. vivipara* stoneflies. a, A female walking on the surface of water, and b, after raising her wings in response to gentle wind. c, A male walking on water, and d, after raising his wings in response to wind. Note the stereotypic leg posture of the stoneflies with raised wings. This leg stance provides a wide and stable base during sailing. e, Head-on view of a female with raised wings. This particular photograph was taken in still air; the stonefly is walking and periodically raising its wings, presumably in response to very small air currents. Although the leg posture is atypical, the wing posture nicely shows the 'spinnaker sail' arrangement of the wings. Video segments in platform-independent QuickTime format can be obtained through the World Wide Web (<http://cac.psu.edu/~jhm10>). f, Drawing of an apterygote insect (Diplura; Campodeidae) to show the gross morphological similarity of sailing stoneflies and primitively wingless insects.



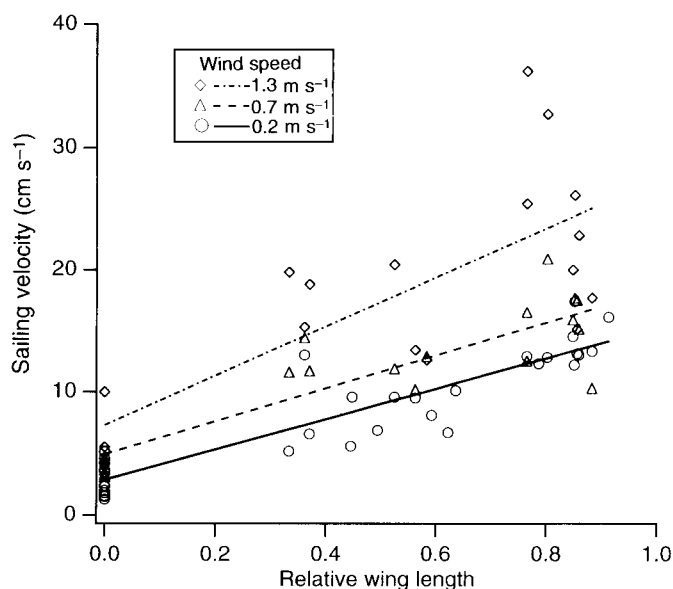


FIG. 2 Sailing velocity of *A. vivipara* stoneflies as a function of relative wing length (forewing length/body length). Each data point represents a different individual. Data from wind speeds of 0.5 and 1.0 m s^{-1} ($N = 4$ for each wind speed) were too few to establish a regression line; these points have been omitted to improve clarity of the figure. Data points at a wing length of zero represent individuals that did not raise their wings in response to wind, thus approximating the aerodynamic performance of wingless stoneflies. Multivariate regression shows that forewing length (partial $r^2 = 0.57$) and wind speed (partial $r^2 = 0.20$) contributed significantly ($P < 0.0001$; $N = 79$) to sailing velocity, whereas body length did not ($P = 0.74$).

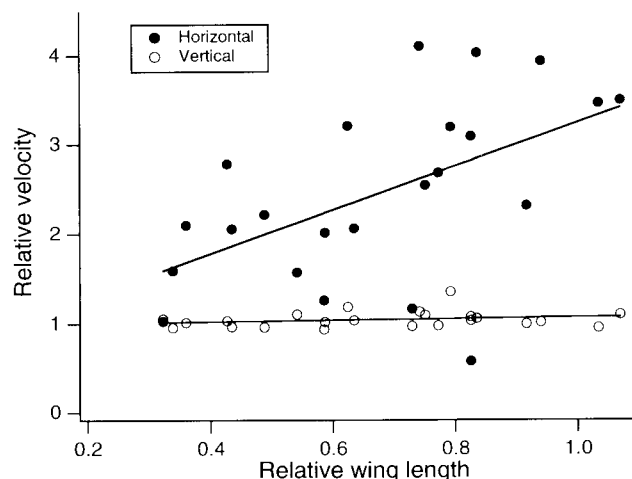
tional advantage of wings for sailing versus gliding. We blew gently on *A. vivipara* stoneflies to stimulate and verify wing raising, then dropped them into still air above a motion-tracking device that recorded their three-dimensional glide path. Each individual was subsequently tested after wing removal. Relative horizontal velocity (velocity with wings/velocity without wings) increased with increasing wing size (Fig. 3). On average, the smallest wings yielded a 1.7-fold improvement in relative terminal horizontal velocity compared to the same individuals after wing clipping. Relative terminal vertical velocity was not affected by wing size.

Relative horizontal velocity was higher for surface sailing than for aerial gliding at all relative wing sizes (Fig. 4), and gliders needed wings 80% as long as their body before they reached the level of increase in relative performance achieved by sailers with the smallest relative wing size (33% of body length). This difference probably stems from the fact that sailing stoneflies maintain a nearly constant and perhaps optimal body orientation with respect to the wind (Fig. 1*b,d,e*), whereas stoneflies in air tend to tumble, with their wings often in a poor position to produce beneficial aerodynamic forces. Thus these data suggest that sailing

has a greater potential to drive the evolution of insect wings than does aerial gliding.

An early trend in insect wing evolution was an elaboration of thoracic winglets, accompanied by a reduction and eventual loss of serially homologous abdominal winglets^{7,8}. The loss of abdominal winglets is difficult to explain in the context of aerial gliding or thermoregulation, for all lateral projections from the body would have contributed to both gliding performance and solar energy capture^{5,6,10}. In contrast, the surface-sailing hypothesis offers a clear explanation for abdominal winglet reduction, based on the effect of abdominal winglets on body orientation. The centre of rotation for a surface sailing insect is located approximately midway between the thoracic wing bases. Aerodynamic forces on thoracic wings would cause little net rotational moment, but forces on abdominal winglets located posterior to the centre of rotation would have a large rotational moment, causing the body to rotate head-on into the wind. In such a position (Fig. 1*e*), abdominal winglets would be downwind of thoracic winglets; they would not increase the aerodynamically functional surface area or contribute significantly to sailing performance.

FIG. 3 Relative velocity (mean terminal velocity with wings/mean terminal velocity without wings, measured from 2–6 tests for each individual before and after the wings were removed completely by clipping) as a function of relative wing length during aerial gliding by *A. vivipara* stoneflies ($N = 23$; each data point represents one individual). Wing length had a significant effect on horizontal velocity ($r^2 = 0.32$; $P = 0.005$) but not on vertical velocity ($r^2 = 0.02$; $P = 0.53$). Absolute terminal vertical velocity varied significantly with body mass ($r^2 = 0.56$; $P < 0.0001$) according to the equation: velocity (m s^{-1}) = -0.072 body mass (mg) $- 1.25$. Absolute terminal horizontal velocity varied significantly ($r^2 = 0.57$; $P = 0.0007$) with wing length according to the equation: velocity (m s^{-1}) = 0.043 wing length (mm) $- 0.015$. Mean body mass and length of *A. vivipara* stoneflies was 2.39 mg (range, 0.7–5.9 mg) and 4.8 mm (range, 2.4–7.4 mm). Aerial motion through a 10 cm $\times$ 8 cm $\times$ 8 cm airspace was tracked in 3 dimensions with a Mac-Reflex motion analysis system (Qualysis Inc., Glastonbury, CT; 30,000 $\times$ 30,000 pixel sensor grids; 60 Hz sampling) modified for 'inverse video', in which the centroid of a dark object (an insect that has not been marked or otherwise modified) is detected as it moves in front of a bright background (fluorescent lights diffused by white plexiglas). Stoneflies were dropped from a height 15 cm above the calibrated airspace, and they reached terminal velocity before or shortly after entering the airspace through which they were tracked.



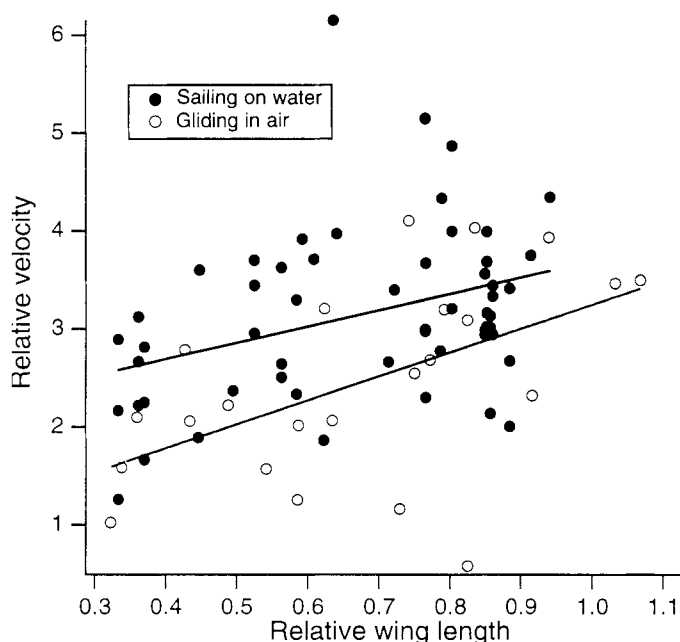


FIG. 4 Relative horizontal velocity (velocity with wings/velocity without wings) for *A. vivipara* stoneflies sailing across water and gliding horizontally through air, as a function of relative wing length (wing length/body length). Relative velocity for sailing was estimated by dividing the sailing velocity of each wing-raising individual (Fig. 2) by the velocity predicted from a multivariate model (independent variables are body mass, body length and wind speed; $r^2 = 0.46$; $P = 0.005$) based on sailing velocity of the 24 individuals that did not raise their wings (data points at zero wing length in Fig. 2). Analysis of covariance indicates that the performance benefit of wings used for sailing is significantly greater than that of wings used for aerial gliding ($P = 0.0013$; covariate equals relative wing length).

The apparent homology of wing venation and wing articulation among all winged insects^{7,8} has been interpreted as powerful evidence that insect flight evolved only once^{3,11,12}, but the use of wings for non-flying surface locomotion shows that wings and flight are not necessarily coupled evolutionarily. Thus evolutionary radiation of early surface-locomotors may have preceded the attainment of powered flight, and therefore flight could have evolved several times, even though all flying insects share the same basic wing venation and articulation. Given this possibility, stoneflies may have retained or reverted to primitive locomotory behaviour and capacity in a form that is little changed from ancestral insects that first evolved wings a third of a billion years ago. At the very least, surface locomotion in stoneflies demonstrates how morphologically primitive insects can use rudimentary wings and wing motions to accomplish aerodynamic locomotion, which in turn suggests new ways to interpret fossils and the evolutionary history of winged insects. □

Unusual thermal defence by a honeybee against mass attack by hornets

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THE giant hornet *Vespa mandarinia japonica* (Hymenoptera: Vespidae) is the only hornet species known to have evolved *en masse* predation of other social bees and wasps. Here we show that hornets is initiated by secretion of a foraging-site marking pheromone from the van der Vecht glands (metasomal sternum VI glands) by a single foraging hornet. The lone hornet rubs the basal tuft of the terminal gastral sternite around a prey food resource, such as a honeybee colony, and the hornet nestmates then congregate and attack the marked site *en masse*. The sympatric Japanese honeybee *Apis cerana japonica* (Hymenoptera: Apidae) can detect the hornet marking pheromone, and responds by increasing the number of defenders at the nest entrance. When an invading hornet is captured by a defending bee, more than 500 other bees quickly engulf the hornet in a ball which contains isoamyl acetate. Thermography showed that the ball temperature is very high ($\sim 47^\circ\text{C}$), which proves lethal to the hornet but not to the bees. Defenders patrolling the nest entrance also generate high temperatures. These findings suggest that aspects of the interaction between *V. mandarinia japonica* and *A. cerana japonica* are specifically coevolved.

The Japanese giant hornet is a major predator of social bees and wasps. Unlike the Japanese honeybee, colonies of the introduced European honeybee *Apis mellifera* are quickly destroyed by mass attacks of the hornet¹, because the giant hornet is allopatric and so the European honeybee has not evolved an effective defence. Earlier observations of the defensive behaviour of the Japanese honeybee suggested that the bees sting the hornet to death², but during our study in Tokyo from 1984 to 1994 we found no evidence of stinging when killed hornets were investigated.

The foraging sequence of the giant hornet exhibits several distinct phases³. First, a lone foraging hornet finds the honeybee nest, kills individual bees and takes them to its nest to feed to the larvae (hunting phase). After several return visits, the foraging hornet marks the site by rubbing its van der Vecht gland on or near the bee colony (recruitment phase; Fig. 1a). Some polistine wasps (*Polistes*, *Mischocyttarus*, *Parapolybia* and some *Ropalidia*) are known to secrete ant repellent from the van der Vecht gland^{3,4} (Fig. 1b), but this is not the case with *Vespa*.

Soon after marking, nestmate hornets flying in the same area congregate at the marked site and start individual hunting. Their hunting behaviour suddenly changes when there are three or more, and they attack *en masse* (slaughter phase). One hornet can kill up to 40 European honeybees per minute with its mandibles, and a colony of 30,000 bees can be killed in 3 hours by a group of 20–30 hornets, which then occupy the hive (occupation phase). During this occupation period, which lasts more than 10 days, the hornets carry bee larvae and pupae to their nest as food for their larvae.

Unlike the European honeybee, the Japanese honeybee has an effective defence against hornet attack. Instead of counter-attacking the hornet individually, more than 100 workers crawl around the nest entrance (Fig. 1c). When a hornet approaches, they simultaneously lift and shake their abdomens and then escape into the nest, and more than 1,000 workers leave the comb and wait just inside the entrance. If a foraging hornet tries

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FIG. 1 Foraging-site marking behaviour of *Vespa mandarinia japonica* (a), (b), and mass defence by *Apis cerana japonica* as a response to the hornet marking pheromone (c).

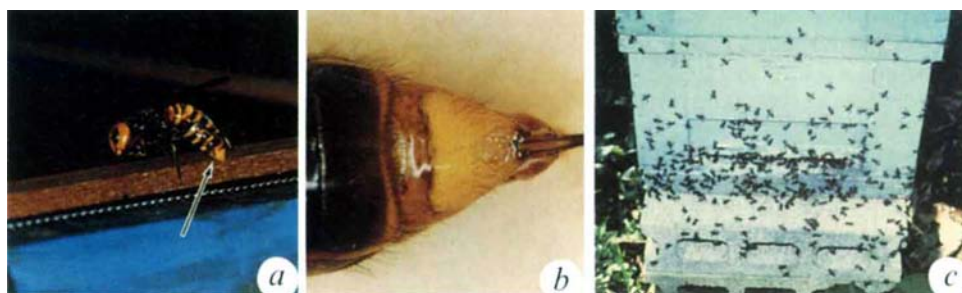
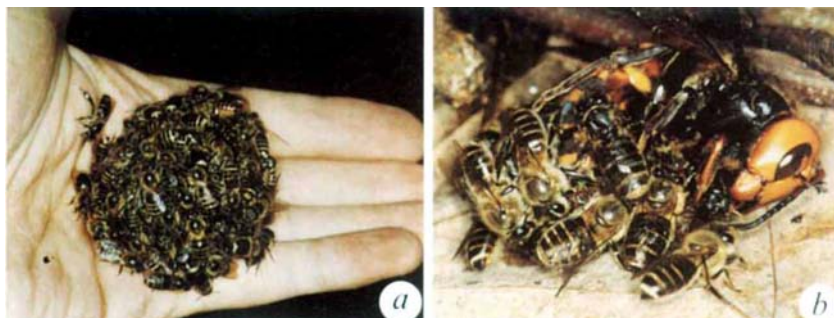


FIG. 2 Defensive ball of *Apis cerana japonica* with ~400 tightly aggregated bees showing no stinging (a) and predator killed by heat (b).



to enter, more than 500 workers quickly engulf it in a ball, the temperature of which rises quickly to 47 °C, which is lethal to the hornet but not to the bees (Fig. 2). In the laboratory, the upper lethal temperature of the Japanese honeybee is 48–50 °C ($n=100$), whereas that of the giant hornet is 44–46 °C ($n=40$). The average duration of balls with an internal temperature above the hornet upper lethal temperature was about 20 minutes ($n=9$). During this time the bees did not move from the middle to the outside of the ball, although several bees killed by the hornet during the struggle are pushed out. When the solitary hunting hornet *V. similima xanthoptera* attacks the Japanese honeybee, a similar ball defence occurs, but with many fewer bees⁵.

A defensive ball formed by *A. cerana japonica* is visualized by thermography (Avio, TVS-8100) in Fig 3a. The thorax temperature of defenders near the nest entrance is already high (Fig. 3b), whereas the thoracic temperature of unagitated fanners at the nest entrance was found to be lower than 35 °C.

An ether extract of three hornet van der Vecht glands impregnated on a filter-paper disc of outside diameter 8mm was placed at the entrance of a Japanese honeybee hive. It causes 50–100 defenders to rush out and begin gnawing the paper, a response to the pheromone that is similar to that during an attack by the hornet. A control filter paper elicited no response. The European honeybee did not react to the impregnated or control filter paper. The Japanese honeybee seems able to detect the hornet foraging-site marking pheromone and responds by luring the first attackers into the nest where they are killed in a defensive ball, thereby reducing the likelihood of subsequent mass attack. The hornet foraging-site marking pheromone is therefore a *V. mandarinia*-specific predator-recognition kairomone for the Japanese honeybee.

How do Japanese honeybees form the defensive ball so quickly? There is some evidence of acoustic and chemical communication. Isoamyl acetate, which is a major alarm pheromone component in the genus *Apis*⁶, was identified in the air surrounding the ball. The heat of the ball may help evaporation of the isoamyl acetate. We do not yet know whether isoamyl acetate is released by the bees in the ball or by bees damaged by the hornet. However, it does appear to alert, and may also recruit nestmates. If the latter is true, this is similar to the enemy-specific recruitment found in the ant *Pheidole dentata*^{7,8}. The defensive

mode evoked by foraging-site marking pheromone lasts for several hours, but the effect of the highly volatile isoamyl acetate should be short lived.

Mass attack by the hornet on other social bees and wasps is only observed in autumn, when hundreds of hornet reproductives (new queens and males) that require large amounts of protein are being reared in the nest. This food pressure may force the hornet into high-risk foraging. Despite the Japanese honeybee's

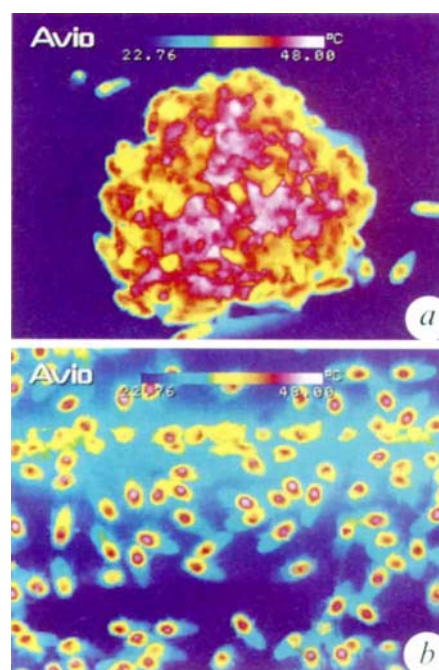


FIG. 3 Colour thermograph showing the 'hot defensive ball' of *Apis cerana japonica* (a) and defender bees crawling around nest entrance (b). The temperature was calibrated using infrared emissivity of the bees' cuticular surface⁹.

sophisticated defensive strategy, the hornet sometimes succeeds in occupying the honeybee nest, if hornet nestmates can reinforce the attack before the first foragers are captured and killed. Occupation by the hornet is often successful when the colony size of the Japanese honeybee is small, although the adult bees escape before the occupation. We believe that the pheromone-regulated mass attack by *V. mandarinia* has evolved to fulfill the need for large amounts of food for reproductives, and as a coevolutionary counter-adaptation to the bee's defensive behaviour.

We have observed a similar mass defence response by the hornet *V. simillima xanthoptera* and the paper wasp *Polistes rothneyi*, both of which can detect the giant hornet foraging-site marking pheromone. Large colonies (600–800 workers) of *V. simillima xanthoptera* exhibit a mass defence to mass attack by the giant hornet. By contrast, instead of counter-attacking, the entire *P. rothneyi* colony escapes and never returns to the original nest; the colony divides, constructs satellite nests, and starts brood rearing again. □

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Blindsight in normal observers

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SOME patients with lesions in visual cortex lack conscious visual experience but, when tested, exhibit a significant ability, termed 'blindsight', to discriminate visual stimuli^{1–3,27}. Here we report two different visual displays that induce blindsight in normal observers. Using an objective measure, we show that conscious experience remains defective at presentation times much longer (1 s) than the onset of visual sensitivity (~60 ms). To obtain this effect, we generate a contrast between visual textures and then conceal the contrast by superimposing 'complementary' textures. Complementarity can involve either opposite motion or binocular rivalry and orthogonal orientation. In both cases, observers locate the texture contrast reliably but do not, by either subjective or objective measures, consciously experience it. Taken together with present knowledge of the visual cortical site(s) at which opposite motion and rivalrous orientation interact^{4–7}, this observation bears upon the functional anatomy of conscious visual experience.

Our paradigm randomly interleaves two matched displays within each block of trials. Both displays contain a target of uniform visual texture and a background of contrasting texture. In one display the target is subjectively visible, whereas in the other it is not (as is evident by inspection). In spite of this difference, performance turns out to be comparable when practised observers locate the target in a forced-choice task.

Our first pair of matched displays uses moving-dot textures (Fig. 1a, b), and target and background differ in that dots in the two regions move along orthogonal trajectories. In one ('paired') display, each dot is paired with a nearby dot (separation <0.1°) of equal and opposite motion. Observers report seeing a uniform texture of incoherently flickering dots⁸ without any impression of a target whatsoever. In the other ('unpaired') display, dots of equal and opposite motion are placed at random distances (<10°) to the original dots. Observers report seeing two coherent sets of dots sliding over each other like two surfaces ('motion transparency')⁸ with the target appearing as smaller surfaces moving orthogonally. After viewing each trial, observers report the target position (1 of 4 possibilities) and rate their confidence that the reported position is correct (scale of 1 to 10). Instruc-

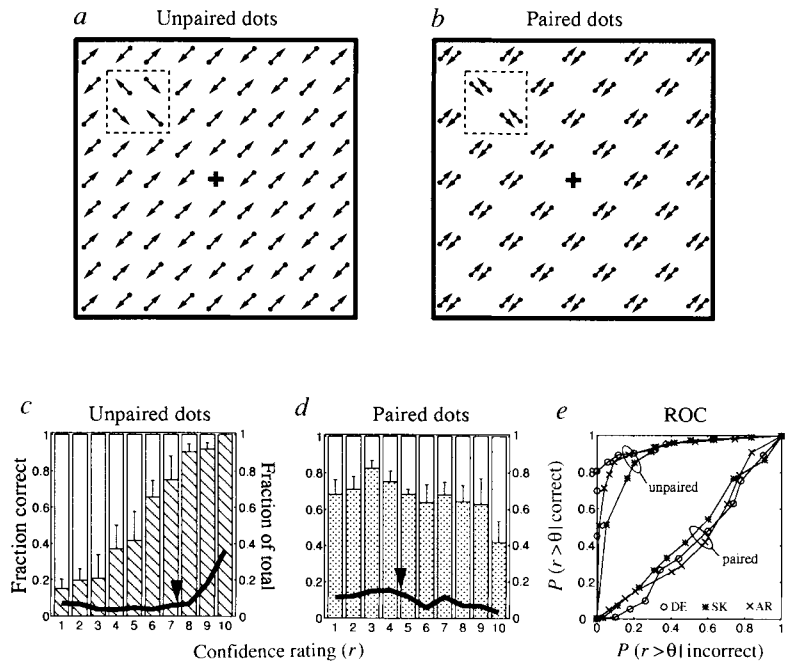
tions encourage observers to use all confidence ratings, independent of whether their overall confidence is high or low. Accordingly, confidence ratings tend to reflect relative rather than absolute confidence levels.

Surprisingly, the percentage of trials in which the position is correctly reported is comparable for paired (69.9 ± 0.9% correct) and unpaired displays (74.4 ± 1.1%) and well above chance (25%) in both cases (presentation time 250 ms). Observers report that they 'guess' target position in paired trials. Confidence ratings document this dissociation of performance and awareness. Figure 1c shows confidence ratings obtained with subjectively visible targets. The distribution is skewed towards higher ratings (Fig. 1c, thick line and arrow) and the fraction of correct responses increases monotonically with confidence (Fig. 1c, bar histogram), exhibiting a highly significant correlation (Kendall's $\tau = 0.99$, $P < 0.0001$). Figure 1d shows the very different outcome obtained with subjectively invisible targets. The distribution is skewed towards lower ratings (Fig. 1d, thick line and arrow) and the fraction of correct responses bears little or no relation to confidence (Fig. 1d, bar histogram, $\tau = -0.61$, $P < 0.05$). Note that instructions tend to minimize any difference in absolute confidence levels. The correlation between the fraction of correct responses and confidence visualized best with the receiver-operating characteristic (ROC)⁹. A highly curved ROC reflects a high degree of correlation and also, it seems, normal subjective awareness of the target (Fig. 1e, unpaired). A straight ROC reflects the absence of a correlation and betrays the lack of subjective awareness of the target (Fig. 1e, paired).

Our second pair of matched displays uses oriented-element textures (Fig. 2a) and target and background differ with respect to element orientation. Observers wear polarizing goggles, permitting independent stimulation of left and right eyes. To ensure spatial register and prevent observers from simply closing one eye, observers discriminate a Landolt C at display centre, a task that is performed best with binocular vision (not shown). In one ('rivalrous') display, corresponding elements impinging upon the left and right eye have orthogonal orientation. Observers report seeing a uniform texture of small 'stars' without any impression of a target whatsoever (presentation time 250 ms). (At longer presentation times, one eye gains dominance and elements presented to the other eye are barred from subjective awareness¹⁰.) In the other ('non-rivalrous') display, corresponding left and right eye elements have the same orientation. Observers report seeing a texture of oriented elements with a contrasting target that is readily apparent. Otherwise, the experimental protocol is the same as with our first pair of matched displays. Again, performance is well above chance for both

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FIG. 1 Psychophysical displays (schematic) with moving-dot textures and results for three observers (D.E., S.K., A.R.; 1,500 trials total, 250 ms, presentation time). Equal and opposite motion conceals a target from visual awareness but does not prevent its localization. Displays are generated on an SGI Indigo 2 (1,024 × 1,024 pixels, subtending $10 \times 10^\circ$, viewing distance 57 cm). Parameters of moving dots are similar to ref. 4 (size, 0.01° ; lifetime, 50 ms; velocity, 2° per second; density, 10 deg^{-2}). Dot trajectories (polar directions $\pi/4$ and $-3\pi/4$ or $3\pi/4$, $\pi/4$ and $-\pi/4$) are orthogonal in the target (size, $2 \times 2^\circ$; centred in one of the four quadrants of the display) and background regions. Observers fixate the centre of the display, report the target position (4-alternative forced-choice), and rate their confidence that the reported position is correct (scale of 1 to 10). *a*, Unpaired display (dots of equal and opposite motion placed at random distances $<10^\circ$) with visible target. *b*, Paired display (dots of equal and opposite motion separated by $<0.1^\circ$) with concealed target. Paired and unpaired displays are randomly interleaved. *c*, *d*, *e*, Analysis of performance and confidence. Let n_r , c_r and i_r be the number of all trials, correct trials, and incorrect trials with confidence rating r , respectively, with $n_r = c_r + i_r$ and $r = 1, \dots, 10$. *c*, *d*, Thick line and arrow: fraction of trials with confidence rating r , γ_r (mean of all observers, $\gamma_r = n_r / \sum_r n_r$). The arrow marks the distribution mean. Bar histogram: fraction of correct responses among trials with confidence rating r , ξ_r (mean and standard error of all observers, $\xi_r = c_r / n_r$). For unpaired displays, the fraction ξ_r increases almost monotonically with the confidence rating (*c*), whereas for paired displays there is no significant relation (*d*). *e*, Receiver-operating characteristics (ROCs) for paired and unpaired trials (individual observers). The ROC of paired trials is essentially straight, showing failed awareness of the target, whereas the ROC of unpaired trials is highly curved, showing normal awareness of the target. (Here,



the ROC is defined as the conditional probability of a confidence rating $r > \theta$, $\theta = 0, 1, \dots, 10$, given that the response is correct, $P_{(r > \theta | \text{correct})}$, plotted against the conditional probability of $r > \theta$ given that the response is incorrect, $P_{(r > \theta | \text{incorrect})}$, where $P_{(r > \theta | \text{correct})} = \sum_{r > \theta} c_r / \sum_r c_r$ and $P_{(r > \theta | \text{incorrect})} = \sum_{r > \theta} i_r / \sum_r i_r$.)

FIG. 2 Psychophysical displays (schematic) with oriented-element textures and results for three observers (D.E., S.K., A.R.; 1,200 trials; 250 ms). Binocular fusion of orthogonally oriented elements conceals a target from visual awareness but does not prevent its localization. Left and right eyes are stimulated dichoptically with the help of polarizing goggles. Both eyes view 20×20 array of oriented elements (2-dimensional Gabor functions with polar orientation $\pm\pi/4$, period 0.448° , $\sigma = 0.224^\circ$, phase $\pm\pi/2$). Elements are oriented orthogonally in the target (size $2 \times 2^\circ$, centred in one of four quadrants of the display) and the background region (for both eyes). To ensure binocular viewing and to control fixation, observers discriminate a small (0.5°) Landolt C at the centre of the displays of both eyes. If the polar position of the opening of the C is reported correctly, the trial proceeds, if not, the trial is aborted. Next, observers report target position and rate their confidence. *a*, Rivalrous display (corresponding left and right elements have orthogonal orientation) with concealed target. Not shown: non-rivalrous display (corresponding left and right eye elements have same orientation) with visible target. Rivalrous and non-rivalrous displays are randomly interleaved. *b*, *c*, Thick line and arrow: fraction of trials with confidence rating r , γ_r (mean of all observers). The arrow marks the distribution mean. Bar histogram: fraction of correct responses among trials with confidence rating r , ξ_r (mean and standard error of all observers). *d*, The ROCs for non-rivalrous trials are highly curved whereas those for rivalrous trials are essentially straight, showing the difference between normal and failed awareness of the target (individual observers).

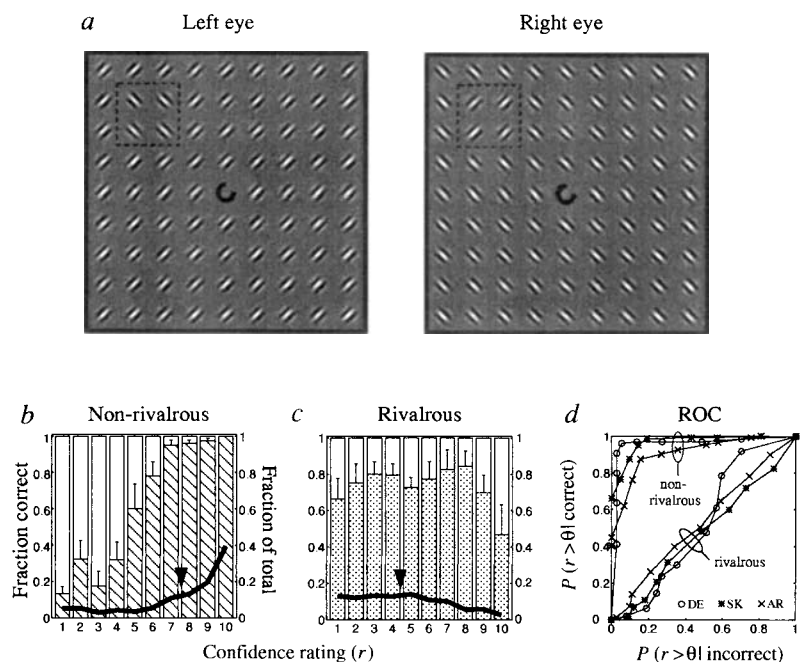
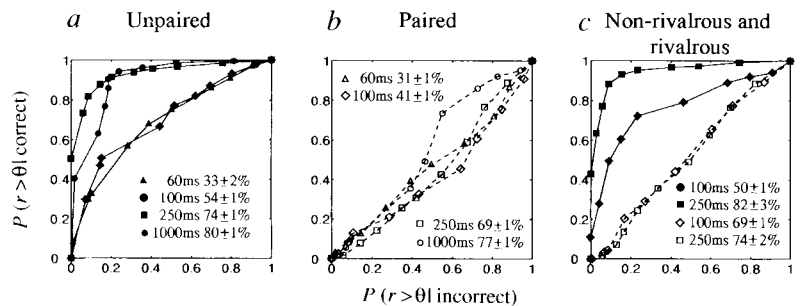


FIG. 3 Receiver-operating characteristics obtained over a 16-fold range of presentation time. Data from Figs 1 and 2 at 250 ms presentation time combined with additional data at 60 ms, 100 ms and 1 s presentation time (2,200 trials; observers D.E., S.K.). ROCs are drawn with different symbols, and insets give the respective presentation time and performance (mean of all observers). When moving-dot displays are presented for 1 s, it is necessary to prevent serial scrutiny of individual display regions. To this end, the orientation of dot trajectories changes every 250 ms in both target and background regions. Oriented-element displays were not investigated at >250 ms because one eye dominates perception at longer presentation times. *a*, *c*, Filled symbols and solid lines: curved ROCs indicate normal awareness of the target even at the lowest levels of performance (unpaired and non-rivalrous displays). *b*, *c*, Open symbols and dashed



lines: essentially straight ROCs indicate failed awareness of the target even at the highest levels of performance (paired and rivalrous displays).

rivalrous ($75.2 \pm 1.1\%$ correct) and non-rivalrous ($83.3 \pm 1.8\%$) displays (presentation time 250 ms), although observers believe themselves to be 'guessing' on rivalrous trials. Again, subjectively visible and invisible targets produce differentially skewed distributions of ratings (Fig. 2*b, c*, thick line and arrow) and the correlation between success and confidence is strong for visible (Kendall's $\tau = 0.90$, $P < 0.0005$) and absent for invisible targets ($\tau = 0.02$, $P < 0.05$) (Fig. 2*b, c*, bar histogram). The curvature of the ROC again highlights this difference (Fig. 2*d*).

The dissociation of performance and awareness in paired and rivalrous displays is not limited to a narrow threshold region but holds over a wide range of performance (Fig. 3). Increasing presentation time from 60 ms to 1 s raises performance from $31 \pm 1\%$ to $77 \pm 1\%$ for paired, and from $33 \pm 2\%$ to $80 \pm 1\%$ for unpaired displays, yet the respective ROCs remain straight, or curved, throughout this range (Fig. 3*a, b*). Similar results are obtained with rivalrous and non-rivalrous displays at 100 ms and 250 ms presentation time (Fig. 3*c*). Even extensive visual practice (>20 sessions of 1 h/day) does not change the outcome (not shown). It appears that, without subjective awareness, the observer cannot gauge signal strength and thus cannot estimate the accurateness of his or her response.

This seemingly complete lack of awareness far above the onset of visual sensitivity warrants comparison with blindsight^{1, 3, 27}. In previous attempts to dissociate awareness and performance in normal observers ('subliminal perception')^{11, 13}, a direct measure (for example, a task like our 4AFC location task) typically shows zero or near-zero visual sensitivity (that is, performance is at or near chance), whereas an indirect measure (for example, priming of a subsequent task) shows that some visual processing has taken place. Accordingly, this situation is not comparable to blindsight. In an explicit attempt to demonstrate blindsight in normal observers, a recent study dissociates detection and localization performance, showing that undetected stimuli are localized somewhat better than chance^{14, 15}. However, it has been argued that this dissociation may simply concern performance and be unrelated to awareness¹⁶. In general, controversy persists about whether performance can be convincingly dissociated from awareness with any paradigm^{16, 19}.

Why do two of our displays induce blindsight and two others support visual awareness of the target? Single-cell evidence from non-human primates who view similar stimuli suggests reduced or altered input to extrastriate cortex as a possible reason. This would imply that our observations and clinical blindsight^{1, 3, 27} may have a similar genesis. In general, striate cortex is thought to exhibit an increased response at the location of a texture contrast, presumably because of the surround inhibition intrinsic to this area^{20, 24}. As opposite motion and rivalrous orientation appear to be processed independently at the level of striate cortex^{4, 7, 25}, this increased response should be evident in direction-selective cells with both paired and unpaired displays and in orientation-selective cells with both rivalrous and non-

rivalrous displays. In extrastriate cortex, however, opposite motions and rivalrous orientations seem to interact in an inhibitory manner: in extrastriate area MT, for example, paired dot textures elicit approximately half the activity (in direction-selective cells) than unpaired dot textures⁴ and, in extrastriate areas V4 and MT, rivalrous patterns elicit significantly less activity than non-rivalrous patterns^{5, 6}.

Assuming that our paired and rivalrous displays impede the direct propagation of activity from striate to extrastriate cortex, the situation resembles damage to striate cortex and clinical blindsight in that extrastriate input is reduced and much of the remaining input is derived from subcortical sources^{3, 27}. Accordingly, a reduced amount or a subcortical source of extrastriate input, or both, may impoverish subjective awareness. This would be consistent with the recent suggestion, on anatomical grounds, that subjective awareness may correlate with neural activity in extrastriate but not striate cortex²⁶. Note that, even if paired and rivalrous displays would completely block the direct striate-extrastriate projection, activity in striate cortex could still affect activity in extrastriate areas indirectly by way of the superior colliculus, the pulvinar, or other subcortical nuclei^{3, 27}. In this way, a texture contrast registered in striate cortex could guide behaviour without entering subjective awareness. □

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Which parts of the road guide steering?

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A DRIVER steering a car on a twisting road has two distinct tasks: to match the road curvature, and to keep a proper distance from the lane edges. Both are achieved by turning the steering wheel, but it is not clear which part or parts of the road ahead supply the visual information needed, or how it is used. Current models of the behaviour of real drivers^{1,2} or 'co-driver' simulators³⁻⁵ vary greatly in their implementation of these tasks, but all agree that successful steering requires the driver to monitor the angular deviation of the road from the vehicle's present heading at some 'pre-view' distance ahead, typically about 1 s into the future. Eye movement recordings generally support this view⁶⁻⁹. Here we have used a simple road simulator, in which only certain parts of the road are displayed, to show that at moderate to high speeds accurate driving requires that both a distant and a near region of the road are visible. The former is used to estimate road curvature and the latter to provide position-in-lane feedback. At lower speeds only the near region is necessary. These results support a two-stage model¹ of driver behaviour.

We used a driving simulator based on program developed on an Archimedes A5000 RISC computer. This provided a perspective view of the moving edges (white on black, as in night driving) of a single-lane road, 3 m wide, with many bends, modelled on a real road used in a previous study of drivers' gaze direction⁶. There was no other scenery. The view extended for 63 m and was viewed from a height of 1.1 m. It was updated at 7 Hz, and was presented on a 60-cm wide screen at a distance of 80 cm, providing a road view with the same angular dimensions as the original. The display was driven by a steering wheel monitored by a 'mouse'. The integration constants converting wheel angle to car direction and car direction to lateral road position were chosen to give as realistic a performance as possible, and drivers found it easy to negotiate the road successfully. After an initial few drives, performance changed little. The measure of accuracy chosen to assess different experimental conditions was the reciprocal of the standard deviation (s.d.) of the car's position relative to the road's centre line, measured over the whole drive (68 s at 16.9 m s⁻¹ (61 km h⁻¹; 38 m.p.h.)). This s.d. was typically 0.1–0.2 m.

The road was viewed either in its entirety, or with only one or more 1° high segments visible, these being positioned in 9 locations between 1° and 10° below the horizon. These segments moved exactly as they would in the full road. Three typical examples of driver performance are shown in Fig. 1. When only the distant part of the road was visible (*a*) the curvature was smoothly matched, but the position-in-lane was not well maintained. With only the near region (*c*) steering was difficult and jerky, as the short available response time forced the driver to change from a smooth to a 'bang-bang' feedback strategy. However, position in lane was quite well maintained compared with *a*. With middle-distance segments (*b*), both curvature and position were dealt with reasonably well, and this region gave the best driving performance. At the fast but not reckless speed of 16.9 m s⁻¹, there is an optimum position for the visible segment 5.5° down from the vanishing point, that is, 11.4 m or 0.68 s ahead (Fig. 2*b*, filled circles). However, the accuracy was 20% lower than with the whole road visible, and at higher speeds that percentage increased. At slower speeds (<12 m s⁻¹), accuracy with the optimum 1° segment was similar to or even better than the performance with the whole road visible; thus competent steering can occur with only a small region of the whole flow-field visible^{10,11}.

At higher speeds a better performance was achieved when two road segments were visible (Fig. 2*b*). The second road segment improved performance dramatically, depending on where it was positioned. With one visible region in the distant part of the road, a second region enhanced accuracy if it lay in the near region (open circles), and vice versa (open squares). However, merely doubling the amount of road edge in the near or far regions, or even near the single-segment optimum at 5.5°, had no effect. With both near and far segments visible the accuracy matched that with the whole road visible (dotted line). At higher speeds (19.7 m s⁻¹), this enhancement effect was even more dramatic, but at lower speeds (<12.5 m s⁻¹) the presence of an extra segment in the far part of the road had little effect on accuracy.

When a near segment is present (open circles) it is immediately clear to drivers that its effect is to provide an indication of position-in-lane, thereby preventing the kinds of error seen in Fig. 1*a*. Eye-movement recordings show that drivers rarely fixate this near region, but view it peripherally whilst tracking the distant part of the road (Fig. 2*a*). With a distant segment present (open squares) the enhancement is due to an improvement in stability. With a single segment the jerkiness of steering increases (Fig. 2*c*, filled circles) at near distances (as in Fig. 1*c*), but the presence of a distant segment (open squares) reduces that instability to the same level as when the whole road is visible.

These observations strongly support a double model of steering, originally proposed by Donges¹. More distant parts of the road provide information about road curvature, perhaps through tangent-point tracking^{5,6}. From Fig. 2*b* the optimum

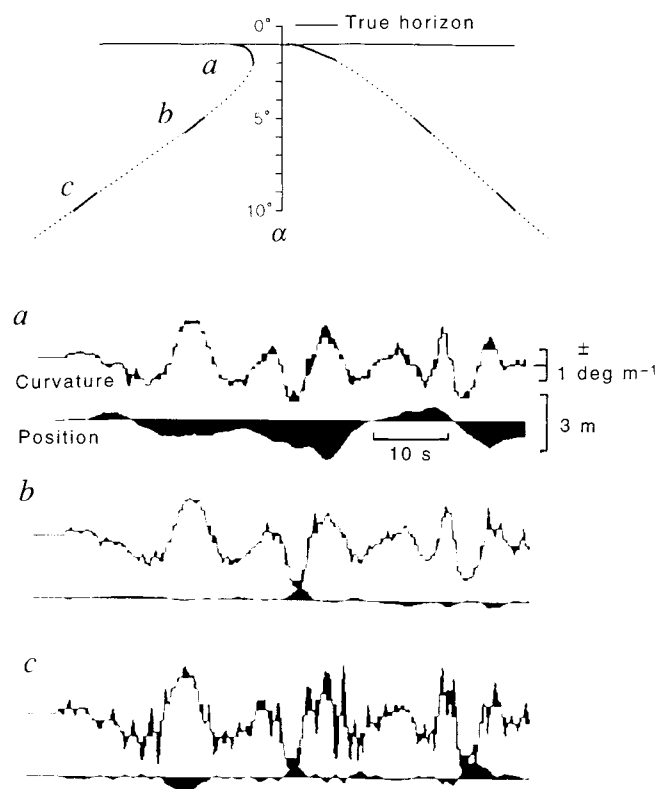


FIG. 1 Typical driver performance with only 1° segments of simulated road visible. The top panel shows locations of segments, the lower panels show corresponding performance. The upper trace in each record gives curvature of both road and vehicle track, with difference between them shown in black. The lower record gives vehicle position relative to centre line of road. In *a* and *b* curvature matching is good and almost smooth, but in *c* it is jerky, indicating instability. In *b*, and to a lesser extent *c*, position-in-lane is well maintained, but in *a* it is very poor. Subject M.L., speed 16.9 m s⁻¹. Road edges white on black background. Details of viewing conditions in text.

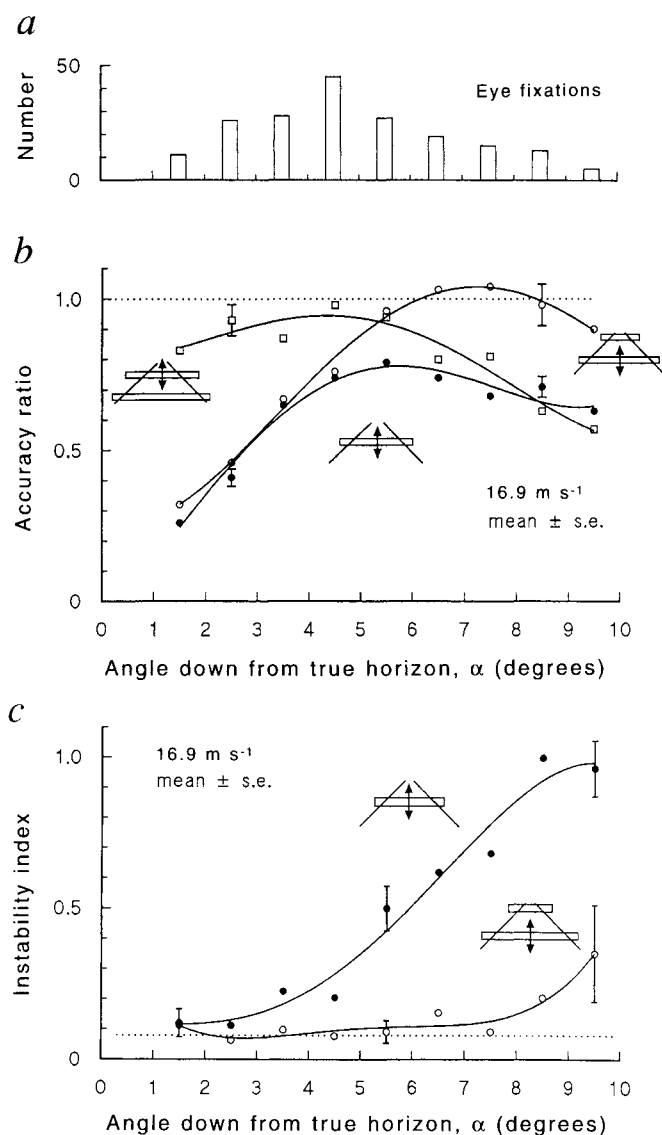


FIG. 2 Measures of driving performance with one and two 1° segments of road visible. In each part the results are combined from 3 drivers (M.L., a 53-year-old male; J.H., a 36-year-old female; and A.M., a 42-year-old male). Speed 16.9 m s^{-1} throughout. a, Histogram of gaze fixation positions on the road measured every second (methods as described previously¹²), when viewing the whole road. Abscissa as in b (α in Fig. 1). b, Accuracy of steering when only one or two 1° segments are visible. With only 1 segment visible (filled circles) there is an apparent optimum 5.5° below the true horizon. With an extra segment in the far part of the road $1-2^\circ$ down (open circles) performance is greatly enhanced, but only if the other segment is in the near part. Similarly a segment in the near part $9-10^\circ$ down enhances performance in the far part (open squares). In the latter case there is an optimum in the distant part of the road about 4° down, which corresponds to the fixation maximum in a. Accuracy is measured as the standard deviation of the distance of the vehicle's track from the centre line of the road, taken over the whole drive ($n \sim 500$). Accuracy ratio is s.d. with the whole road visible divided by the s.d. with one or two segments visible. Dotted line shows the performance with the whole road. Inserts indicate which visible segments are fixed, and which take the positions shown on the abscissa (arrowed). Differences between single and paired regions, at the points where the s.e.s are given, are highly significant ($P < 0.001$, t-test). Points represent means of 5 drives by 3 drivers. Curves are fitted 4th-order polynomials. c, Instability of steering with one region visible (filled circles), and the same region plus an additional region in the far part of the road (open circles). The effect of the far region is to reduce instability to the same level as when the full road is present (dotted line). The addition of a near visible region (not shown) does not reduce instability. The 'instability index' is the product of the number and amplitude of spike-like steering movements, of the kind visible in Fig. 1c, normalized to their maximum value. Other details are as in b.

for this mechanism at 16.9 m s^{-1} lies about 4° below the horizon (0.93 s or 15.7 m ahead). However, accurate position-in-lane information comes from the nearer part of the road, about 7° down (0.53 s or 9.0 m ahead). At moderate to high speeds, the near-road system is unstable on its own, but when road curvature has already been anticipated by the far-road mechanism, the near-road mechanism only needs to fine-tune the system, which can now be done smoothly. At slower speeds the near-road mechanism is adequate on its own, rather like driving in fog. □

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Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator

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NEURONAL degeneration in the hippocampus, a region of the brain important for acquisition of memory in humans, occurs in various pathological conditions, including Alzheimer's disease, brain ischaemia and epilepsy. When neuronal activity is stimulated in the adult rat and mouse hippocampus, tissue plasminogen activator (tPA), a serine protease that converts inactive plasminogen to the active protease plasmin, is transcriptionally induced^{1,2}. The activity of tPA in neural tissue is correlated with neurite outgrowth³, regeneration⁴ and migration⁵, suggesting that it might be involved in neuronal plasticity. Here we show that tPA is produced primarily by microglia in the hippocampus. Using excitotoxins to induce neuronal cell loss, we demonstrate that tPA-deficient mice are resistant to neuronal degeneration. These mice are also less susceptible to pharmacologically induced seizures than wild-type mice. These findings identify a role for tPA in neuronal degeneration and seizure.

Although the expression of tPA in the hippocampus⁶ and its induction by pharmacological and electrical stimulation are well documented^{1,2}, its role in the brain has not been established. As the four different cell types in the hippocampus—neurons, oligodendrocytes, astrocytes and microglia—perform distinct functions that each might involve proteolytic activity⁷, defining the site(s) of synthesis of tPA is an important step in determining its function.

In the mouse, tPA messenger RNA distribution coincides with the neuronal cell layers of the CA1–CA3 hippocampal subfields and the dentate granule cell layer⁶. However, the intimate association of neurons with their surrounding glia has made it difficult to determine which cell types express tPA.

Transgenic mice (tPA/*lacZ*) have been generated that carry a mouse tPA promoter fused to the bacterial *lacZ* gene². The expression pattern of β -gal in these mice² generally reproduced endogenous tPA mRNA hippocampal expression both in location⁶ and in transcriptional activation after induction of seizure¹. The resolution of β -gal staining made it possible to analyse brain sections for tPA promoter activity in great detail. The hippocampal, cytoplasmic β -gal expression showed a punctate pattern within the neuronal cell layers of the hippocampus (Fig. 1*b, c*) and dentate gyrus. However, the stained cells were smaller than the pyramidal cell bodies, suggesting that a subset of microglia surrounding the neurons (satellite microglia) were expressing β -gal and, by inference, tPA mRNA.

To discriminate between neuronal and microglial expression of β -gal, we eliminated neurons in tPA/*lacZ* mice by unilateral intracerebral injection of kainic acid (KA), a glutamate excitotoxic analogue that causes neuronal degeneration. To assess cell death, brain sections were examined by cresyl violet staining of neuronal cell bodies. Consistent with previous reports⁸, complete loss of neurons in the CA1–CA3 pyramidal subfields was observed on the ipsilateral (injected) side (Fig. 1*a*). Granule cells of the dentate gyrus were unaffected by the injection of KA. There was no noticeable degeneration on the contralateral (un-

injected) side⁸. When adjacent tissue sections were stained for β -gal, activity on the ipsilateral side lacking neurons (Fig. 1*b*) was comparable to that on the unaffected, contralateral side (Fig. 1*c*) in the CA1–CA3 subfields. The persistence of β -gal staining in the absence of neurons demonstrates tPA promoter activity in non-neuronal cells.

To test the possibility that missing promoter elements or differences in reporter gene protein expression was obscuring details of tPA localization, we used a histochemical assay for tPA⁶ to visualize endogenous enzyme expression in wild-type mice after excitotoxin-induced neuronal degeneration. The results demonstrated that tPA activity was similar on the two sides of the hippocampus, even though the injected side lacks neurons in CA1–CA3 subfields (Fig. 1*d*). This indicates that tPA is produced primarily by non-neuronal cells, presumably microglia.

Microglia are non-neuronal, macrophage-like cells present in the developing and adult central nervous systems. Upon neuronal injury, microglia are transformed from a resting to an activated state, characterized by changes in morphology, immunophenotype, migration and proliferation^{8,9}. Activated microglia participate in the phagocytosis of neurons and, furthermore, microglial proteases are involved in neuronal degradation¹⁰.

We therefore investigated whether microglial tPA plays a role in the degradation of neurons in the hippocampus. Mice homozygous for a tPA deficiency¹¹ (*tPA*^{-/-}), the two inbred control strains (C57 and 129) used to generate the *tPA*^{-/-} animals, and

FIG. 1 tPA/*lacZ*-expressing cells and tPA enzymatic activity persist after excitotoxin-induced neuronal loss. Low-magnification photomicrograph of coronal sections through the hippocampus stained with cresyl violet illustrates the lesion generated by 1.5 nmol kainic acid (KA) on the injected side (ipsilateral, arrow), whereas contralateral to the lesion (uninjected side) no neuronal death was observed. *a*, Hippocampus from tPA/*lacZ* mouse 5 days after the injection. CA1, CA2 and CA3 denote the hippocampal subfields, DG the dentate gyrus. *b*, tPA/*lacZ* mouse: higher magnification of the ipsilateral side, 16 days post-injection. *c*, tPA/*lacZ* mouse: higher magnification of the contralateral side, 16 days post-injection. Note the persistence of the β -gal staining on the ipsilateral side where the pyramidal cells have been destroyed. Given that neuronal cell death is observed within 12 h post-injection, and the mice were examined at 16 days, it is unlikely that the β -gal staining represents residual, phagocytosed neuronal debris. Scale bar, 20 μ m. *d*, tPA histochemical assay⁶ on a coronal brain section of wild-type mouse injected unilaterally with kainic acid as in *a*. Note the zone of proteolysis indicating tPA enzymatic activity primarily over the CA2–CA3 hippocampal subfields and DG⁶. The arrow points to the zone of proteolysis surrounding the injection track, where microglia accumulate (see Fig. 3).

METHODS. Adult male mice, weighing approximately 25 g, were injected intraperitoneally with atropine (0.6 mg per kg body weight) and then were deeply anaesthetized with metophane. They were placed in a stereotaxic apparatus, and injected unilaterally with 1.5 nmol kainic acid in 0.3 μ l PBS into the hippocampus⁸. The coordinates of the injection were: bregma 2.5 mm, medial–lateral 1.7 mm, and dorsoventral 1.6 mm. The excitotoxin was delivered over 30 s, with the pipette maintained in place for an additional 2 min to prevent reflux of fluid. After a recovery period of 5 (*a*) or 16 days (*b, c*), the animals were anaesthetized and perfused through the heart with PBS followed by 4% paraformaldehyde. The brains were removed and placed in 30% sucrose in fixative overnight at 4 °C. Coronal tissue sections (30 μ m) were prepared, mounted onto slides, dehydrated through increasing gradients of ethanol solutions, and stained with cresyl violet, which stains neuronal cell bodies (*a*); *b* and *c* were stained overnight for β -gal activity and counterstained with neutral red¹⁶. *d*, For the tPA enzymatic activity assay, wild-type mice injected with KA as above were killed 5 days after the injection. The brains were frozen and processed as described previously⁶, except that amiloride was not included in the overlay mixture. The photograph was taken under dark-field illumination after 2 h incubation at 37 °C.

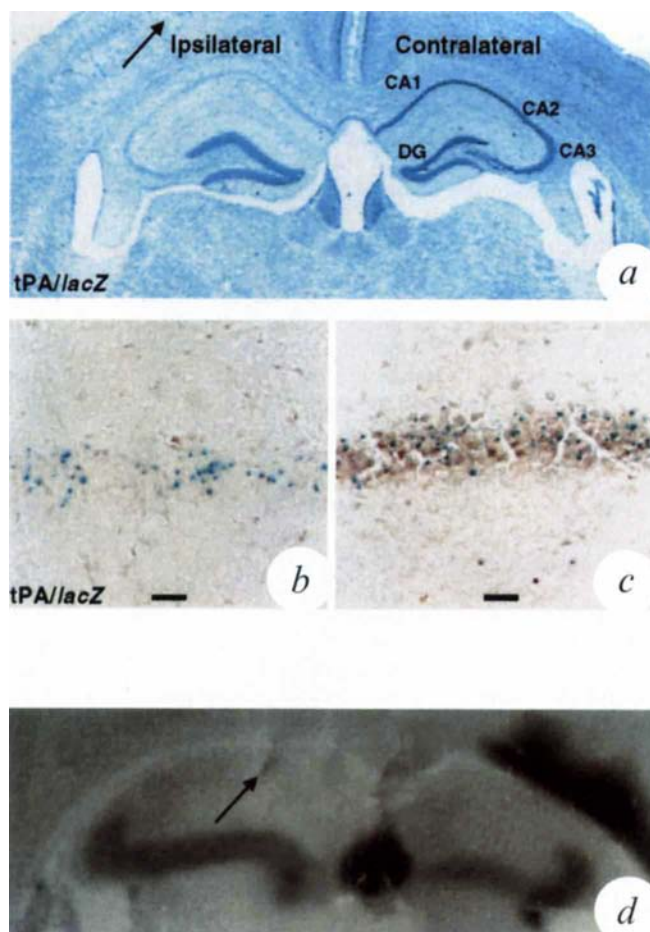


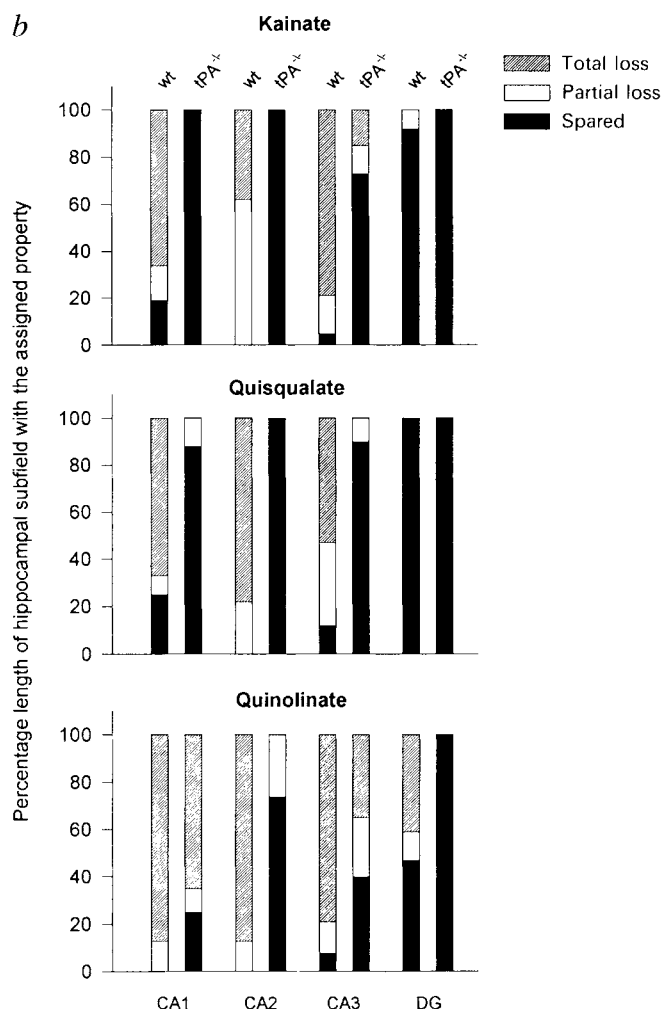
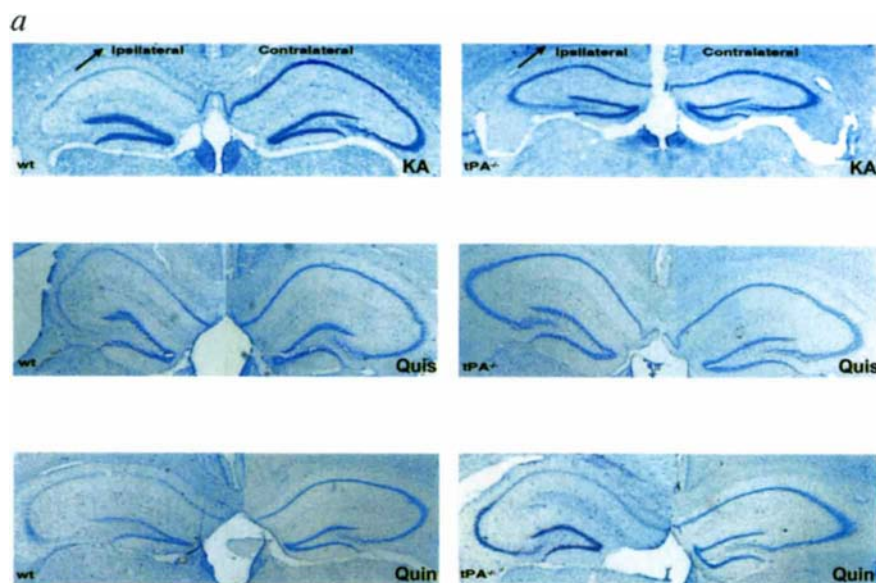
FIG. 2 *tPA*^{-/-} mice are resistant to excitotoxin-induced neuronal degeneration in the hippocampus. *a*, Low-magnification cresyl violet staining of coronal sections through the hippocampus. Excitotoxins, KA (1.5 nmol), quisqualate (Quis, 20 nmol) and quinolinate (Quin, 120 nmol) were injected as in Fig. 1. The brains of the injected mice were analysed 5 days post-injection. Left, wild-type mice; right, *tPA*^{-/-} mice. Compare the destruction of the neurons in the CA fields in the control mice to the extent of their survival in *tPA*^{-/-} mice. The experiment was repeated with eight C57 mice, four 129 mice and 12 *tPA*^{-/-} mice for KA; two C57 and two *tPA*^{-/-} mice for Quis; and two C57 and two *tPA*^{-/-} mice for Quin. *b*, Quantification of excitotoxin-induced neuronal loss in wild-type and *tPA*^{-/-} mice. Two wild-type (wt) and two *tPA*^{-/-} mice for each excitotoxin were injected and the tissue was processed as above. Serial sections (30 μ m) were prepared and stained with cresyl violet. Five or six matched sections from the dorsal hippocampus of wild-type (wt) and *tPA*^{-/-} mice were drawn with a camera lucida and subjected to quantitative analysis; the linear distances of spared (intact; black), partly lost (white) and totally lost (grey) pyramidal cell layer were determined on each section. Distances were digitized from the camera lucida drawings of the hippocampus. The measurements for each category over each hippocampal region were averaged across subjects in a group and plotted.

tPA^{-/-} heterozygous mice were injected with KA. In control and *tPA*^{+/+} animals, essentially complete neuronal loss was observed in the CA1-CA3 subfields, similar to *tPA*^{-/-} mice (strain 129, wild type; Fig. 2*a*, top left; strain C57 and *tPA*^{+/+}, not shown). In contrast, neuronal degeneration in the hippocampus of *tPA*^{-/-} mice was minimal (*tPA*^{-/-}, top right of Fig. 2*a*). There was some cell loss in the immediate area of the injection site, suggesting that the lack of tPA increases the threshold necessary to effect degeneration. These results implicate tPA in the neuronal disappearance induced by KA.

KA is an agonist of one subtype of the glutamate receptor system, but there are other classes of glutamate gated ion-channels⁷: (2-aminomethyl)phenylacetic acid (AMPA) and *N*-methyl-D-aspartate (NMDA). To determine whether the lack of neuronal loss observed was specific for the KA subtype of glutamate receptors, we injected intracerebrally agonists whose action is mediated through the AMPA or NMDA receptors. Resistance to cell death was observed in *tPA*^{-/-} mice after injection of quisqualate (AMPA receptors; Fig. 2*a*, middle) or quinolinate (NMDA receptors; Fig. 2*a*, bottom). Thus the decrease in neuronal cell death in *tPA*^{-/-} mice is observed with a wide range of excitotoxins.

The extent of neural protection in *tPA*^{-/-} mice was determined by quantifying excitotoxin-mediated neuronal loss in the hippocampal subfields (Fig. 2*b*). The linear distance of spared, partly degenerated, or completely lost neurons was measured in matched coronal sections at different rostrocaudal levels of the dorsal hippocampus. In each case the absence of tPA conferred dramatic resistance. For example, in KA-injected animals, C57 control mice demonstrated 66% loss of CA1, 38% loss of CA2, and 77% loss of CA3 neurons. Matched sections of *tPA*^{-/-} mice showed no neuronal loss in CA1 or CA2, and only 15% loss of the CA3 neurons. Similar levels of resistance were observed with quisqualate and quinolinate.

The lack of neuronal degeneration in excitotoxin-injected *tPA*^{-/-} mice could be due to a failure of microglial cell activation. To investigate this, brain sections from KA-injected animals were immunostained for the activated microglia-specific antigen F4/80 (ref. 12). Qualitatively, microglia from wild-type⁸ and *tPA*^{-/-} mice behaved similarly: the cells were activated around the injection site and along the pyramidal fields of CA1-

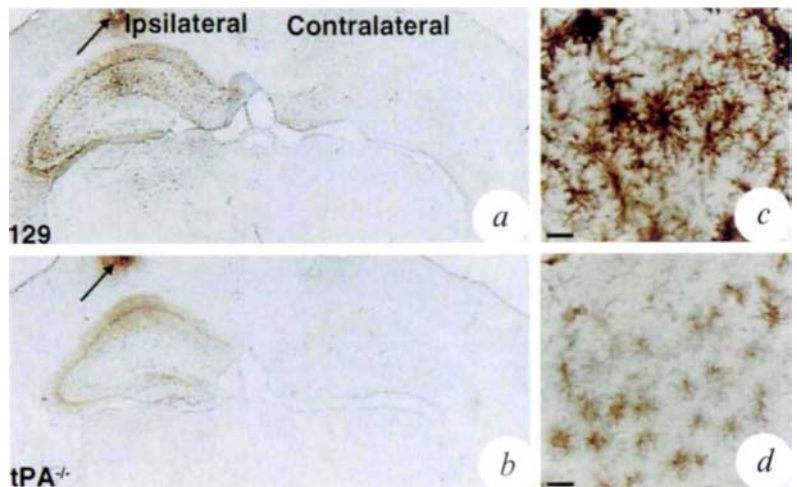


CA3, whereas reduced activation was observed on the uninjected side (Fig. 3*a, b*).

In the hippocampus of wild-type mice, resident microglia have radially oriented processes¹². After injection of KA, the microglial cell number increases and their processes become thickened and more arborized⁸. When the activation of microglia in

FIG. 3 Microglia in $tPA^{-/-}$ mice become activated after injection of kainic acid. Low-magnification F4/80 immunostaining of coronal sections through the hippocampus 5 days post-injection. *a*, 129 mouse; *b*, $tPA^{-/-}$ mouse. Extensive activation is observed on the ipsilateral side and around the injection site (arrow). Microglia on the contralateral side are also activated, but to a much lower level. *c*, High-magnification photomicrograph of representative activated microglia in the CA1 field of stratum radiatum on the ipsilateral side of a 129 mouse; *d*, high-magnification photomicrograph of activated microglia of $tPA^{-/-}$ mouse. Scale bar, 20 μ m.

METHODS. The kainic acid injection was performed as in Fig. 1. The brain sections of the injected mice were incubated with antibodies to the mature macrophage/microglia-specific antigen F4/80 (Harlan Bioproducts) at the dilutions recommended by the suppliers. Biotinylated secondary antibodies were used (Vector) and the ABC reaction was performed (Vector) according to the supplier's protocol.



$tPA^{-/-}$ mice was evaluated using these morphological criteria, an attenuation was observed in $tPA^{-/-}$ mice compared to 129 (Fig. 3*c, d*) and C57 mice (data not shown). These results suggest that tPA could be involved in the activation pathway of microglial cells, which in turn might be related to the degeneration process.

After intracerebral injection of KA, control mice characteristically underwent tonic-clonic seizures in the immediate post-operative period¹³, but $tPA^{-/-}$ mice did not, at the same dose of KA. Therefore, we determined the response of $tPA^{-/-}$ mice to increasing concentrations of seizure-inducing agents. Metrazol, a convulsant that acts through a GABA receptor and increases the transcription of tPA in the hippocampus^{1,2}, was injected intraperitoneally in $tPA^{-/-}$ and control mice. The $tPA^{-/-}$ mice had a higher threshold for metrazol-seizure induction than control strains (Fig. 4*a*). A comparable seizure resistance was observed when KA was injected intraperitoneally in $tPA^{-/-}$ and control mice (Fig. 4*b*). This resistance to seizure was evident both with respect to the dose of metrazol or KA required, and the time delay between drug delivery and onset of seizure (not shown).

Our data show that $tPA^{-/-}$ mice are resistant to neuronal degeneration and seizure after excitotoxin injection. As the brains of $tPA^{-/-}$ mice display no obvious abnormalities¹¹, this phenotype is probably due to an acute lack of tPA expression in the adult hippocampus. However, embryonic development in the absence of tPA might cause subtle alterations in the cytoarchitecture or circuitry of the brain, which could result in the observed effects.

Activated microglia can induce neuronal injury¹⁴, and inhibitors of microglial proteases can extend neuronal survival after axotomy¹⁰. The NMDA receptor antagonist MK-801 blocks

both microglial activation⁹ and the transcriptional induction of tPA¹. The correlation in $tPA^{-/-}$ mice between attenuated microglial activation and resistance to neuronal degeneration further suggests that these two properties may be related. In summary, the available evidence indicates that microglia are central to the degeneration process.

As lack of tPA confers resistance to experimentally induced neuronal degeneration and seizure, tPA activity might contribute

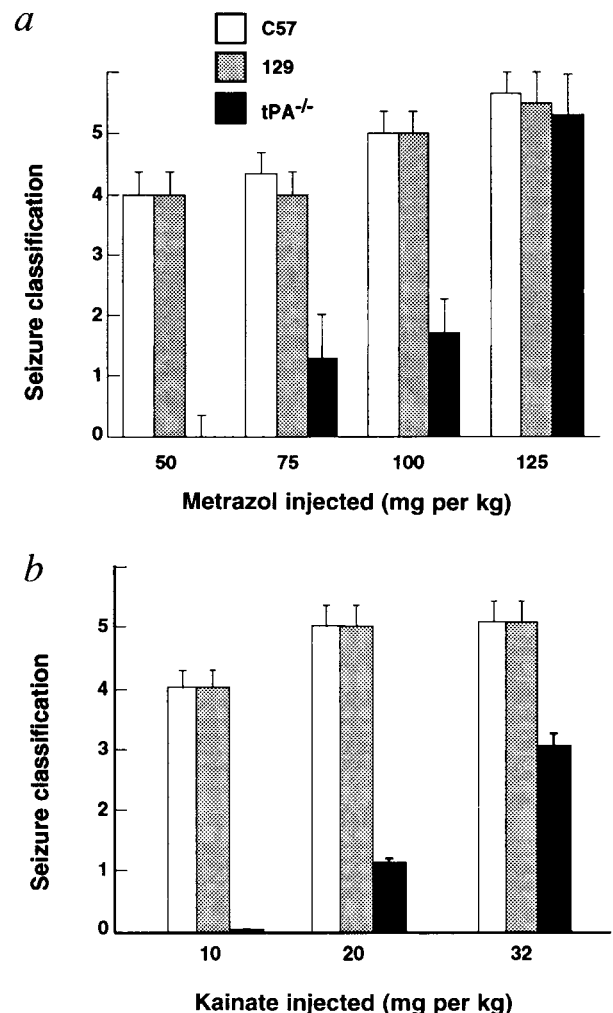


FIG. 4 $tPA^{-/-}$ mice are resistant to pharmacologically induced seizure. Classification of convulsive behaviour of mice injected with *a*, metrazol and *b*, KA. Seizures were categorized as follows¹⁷: 1, arrest of motion; 2, myoclonic jerks of the head and neck, with brief twitching movements; 3, unilateral clonic activity; 4, bilateral forelimb tonic and clonic activity; 5, generalized tonic-clonic activity with loss of postural tone, often resulting in death.

METHODS. Metrazol or KA were injected intraperitoneally at the indicated concentrations. Convulsive behaviour was observed within 5 min, for metrazol, or 2–4 h for KA, from the time of injection in the C57 or 129 mice. The onset of seizure for $tPA^{-/-}$ mice was usually approximately 15–20 min after metrazol delivery. The labels indicating the mouse strains had been removed from the cages and replaced by numbers. The behaviour of the mice was monitored by non-biased, 'blind' judges.

to pathologies associated with accelerated neuronal destruction. In this respect, Alzheimer amyloid β -peptide analogues can stimulate tPA activity *in vitro*¹⁵ and activate microglia in culture¹⁴, implying that elevated microglial protease activity could contribute to Alzheimer's disease. Such reasoning suggests that inhibitors of tPA might have therapeutic potential for treating diseases involving neuronal degeneration and/or seizure. $\square$

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Modulation of GABA_A receptors by tyrosine phosphorylation

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γ -AMINO BUTYRIC acid type-A (GABA_A) receptors are the major sites of fast synaptic inhibition in the brain. They are presumed to be pentameric heteroligomers assembled from four classes of subunits with multiple members: α (1–6), β (1–3), γ (1–3) and δ (1)^{1–5}. Here, GABA_A receptors consisting of $\alpha 1$, $\beta 1$ and $\gamma 2L$ subunits, coexpressed in mammalian cells with the tyrosine kinase vSRC (the transforming gene product of the Rous sarcoma virus), were phosphorylated on tyrosine residues within the $\gamma 2L$ and $\beta 1$ subunits. Tyrosine phosphorylation enhanced the whole-cell current induced by GABA. Site-specific mutagenesis of two tyrosine residues within the predicted intracellular domain of the $\gamma 2L$ subunit abolished tyrosine phosphorylation of this subunit and eliminated receptor modulation. A similar modulation of GABA_A receptor function was observed in primary neuronal cultures. As GABA_A receptors are critical in mediating fast synaptic inhibition, such a regulation by tyrosine kinases may therefore have profound effects on the control of neuronal excitation.

Tyrosine phosphorylation of GABA_A receptors was analysed in human embryonic kidney cells^{5,7} (A293; CRL 1573) expressing GABA_A-receptor $\alpha 1$, $\beta 1$ and $\gamma 2L$ subunits. Receptors composed of these subunits can reproduce many of the properties of neuronal GABA_A receptors^{1,3,5,7}. Tagging of the $\gamma 2L$ subunit with the 9E10 epitope⁸ was used to aid the immunopurification of GABA_A receptors. Addition of this epitope to the $\gamma 2L$ subunit seemed to be functionally silent (S.J.M. and T.G.S. unpublished observations). The $\gamma 2L^{(9E10)}$ subunit was identified

TABLE 1 Effect of cSRC and sodium vanadate on GABA single-channel properties

Channel parameters	Control	+cSRC/NaVo ₃
Mean open time, τ_o (ms)	5.14±1.26 (5)	8.03±1.93* (4)
Open time, τ_{o1} (ms)	2.55±0.76 (5)	3.9±1.15
Open time, τ_{o2} (ms)	13.07±3.48 (5)	13.45±4.88 (4)
Single-channel current, i (pA)	1.79±0.08 (5)	1.73±0.19 (4)
Open probability (%)	0.12±0.09 (5)	0.45±0.19* (4)

Effects of cSRC (75 U ml⁻¹) and sodium vanadate (100 μ M) on single GABA ion-channel parameters. Numbers in parentheses represent the number of experiments.

* Parameters are significantly different ($P<0.05$) when analysed with a modified two-tailed unpaired t-test, allowing for unequal variances.

as a protein of relative molecular mass 42,000 (M_r 42K) that assembled efficiently with the $\alpha 1$ (52K) and $\beta 1$ (56K and 58K) subunits (Fig. 1a, lanes 1–3) as judged by coprecipitation with 9E10 antibodies. The identity of the $\alpha 1$ and $\beta 1$ subunits was confirmed by immunoprecipitation under denaturing conditions, using subunit-specific antisera^{9,10} (Fig. 1a, lanes 4–7). The multiple bands for the $\alpha 1$ and $\beta 1$ subunits seemed to result from proteolysis^{9,10}. In native immunoprecipitations, a protein of approximately 76K (Fig. 1a, lanes 1–3) coprecipitated with the GABA_A receptor subunits. This was identified as the molecular chaperone, heavy-chain-binding protein (C. Connolly and S.J.M., unpublished observations).

To analyse tyrosine phosphorylation, A293 cells were co-transfected with complementary DNAs for GABA_A receptor subunits and a cDNA encoding vSRC, the cellular homologue of which is widely expressed in the central nervous system^{11,12}. Western blotting demonstrated that A293 cells have low levels of phosphotyrosine, which could not be significantly enhanced by treatment with vanadate, a tyrosine phosphatase inhibitor (Fig. 1b, lanes 1, 2), suggesting that A293 cells have high intrinsic phosphatase and/or low tyrosine kinase activities^{13,14}. Expression of vSRC in these cells led to a significant increase in phosphotyrosine content (Fig. 1b, lanes 3–5). In accordance with this, receptors composed of $\alpha 1$, $\beta 1$ and $\gamma 2L^{(9E10)}$ subunits, expressed alone in A293 cells, showed undetectable or only low levels of basal phosphorylation^{9,10} (Fig. 1c, lane 5). Coexpression of receptor subunits with vSRC resulted in phosphorylation of the $\gamma 2L$ subunit (Fig. 1c, lane 2) on tyrosine residues only (Fig. 1d, lane 1). The $\beta 1$ subunit was also phosphorylated but to a much lower stoichiometry (approximately eightfold). The predicted major intracellular domain of the $\gamma 2L$ subunit contains a consensus site for tyrosine phosphorylation centred on tyrosines 365 and 367 (refs 6, 12, 15). Mutation of both of these residues to phenylalanines abolished phosphorylation of the $\gamma 2L^{(9E10)}$ subunit when it was coexpressed with the $\alpha 1$ and $\beta 1$ subunits (Fig. 1c, lane 3). Mutation of these sites on the $\gamma 2L^{(9E10)}$ subunit led to an increased phosphorylation of the $\beta 1$ subunit (Fig. 1c, lane 3) which occurred exclusively on tyrosine residues (Fig. 1d, lane 2). The phosphorylation of the $\beta 1$ subunit was abolished by mutation of tyrosines 384 and 386 within the predicted intracellular domain of this subunit (Fig. 1c, lane 4).

The functional effects of tyrosine phosphorylation were determined by whole-cell recording in A293 cells. Intracellular application of the tyrosine kinase inhibitor genistein to cells expressing vSRC and $\alpha 1\beta 1\gamma 2L$ receptor subunits led to a time-dependent decrease in the amplitude of the GABA response (Fig. 2a). Biochemical analysis of $\alpha 1\beta 1\gamma 2L$ GABA_A-receptor subunits exposed to genistein also revealed a 10–20% decrease in the phosphorylation of the $\gamma 2L$ subunit (data not shown). Genistein, however, had no effect on receptors incorporating the mutant $\gamma 2L^{Y(365/367)F}$ subunit (Fig. 2b). Expression of this mutant subunit did not differ significantly from that of the wild-type

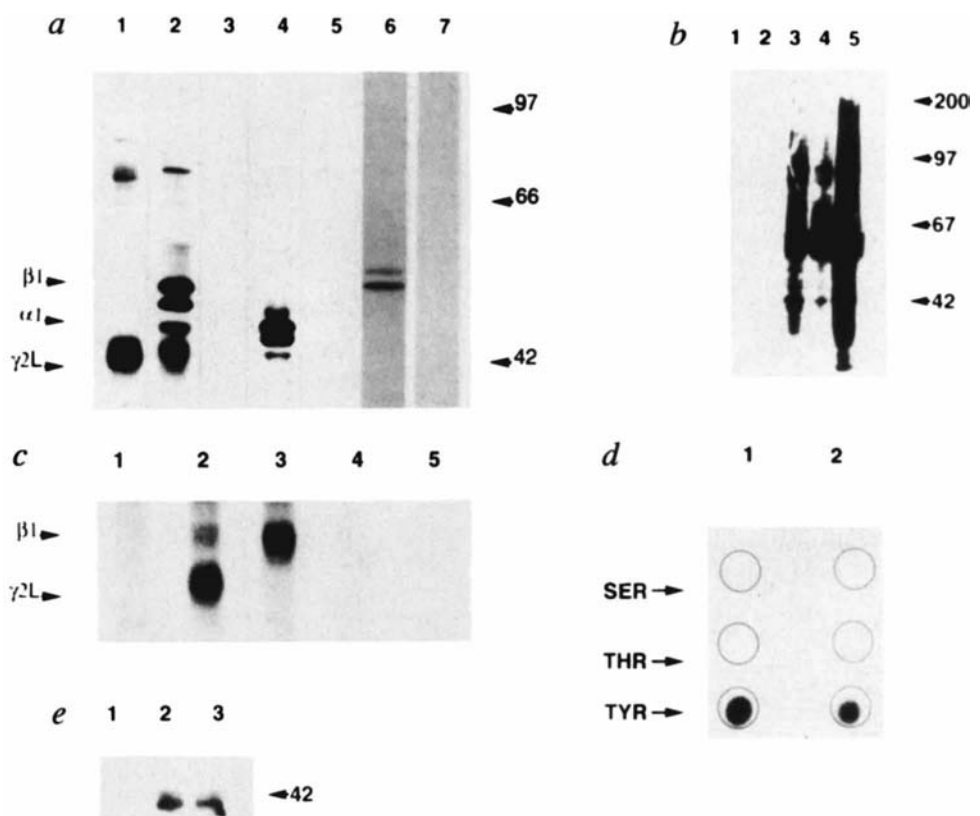
$\gamma 2L^{(9E10)}$ subunit, as judged by western blotting (Fig. 1e) and receptor pharmacology (data not shown). Modulation of GABA_A-receptor function was also studied by exposing receptors expressed alone in A293 cells to purified cSRC by means of intracellular dialysis. In cells exposed to both cSRC and sodium vanadate, an enhancement of the GABA-activated whole-cell current was evident (Fig. 2c, e). The current mediated by GABA_A receptors incorporating the mutant $\gamma 2L^{Y(365/367)F}$ subunit was not enhanced by cSRC and sodium vanadate (Fig. 2d, e). In common with the vSRC cotransfection experiments, we found no functional effects of phosphorylating the two tyrosine residues in the $\beta 1$ subunit, as the effects of cSRC were totally abolished by mutation of the phosphorylation sites on the $\gamma 2L$ subunit.

To assess whether native neuronal GABA_A receptors are regulated by tyrosine phosphorylation, recordings were made from cultured superior cervical ganglion (SCG) neurons. GABA_A receptor function is modulated in these neurons by benzodiazepines, suggesting the presence of GABA_A receptors incorporating $\gamma 2$ and/or $\gamma 3$ subunits^{6,7,16-18}. Exposure to genistein by means of intracellular dialysis led to a time-dependent decrease in the amplitude of the GABA response in SCG neurons (Fig. 3a, d). Intracellular application of sodium vanadate alone caused an increase in the magnitude of the GABA response, suggesting that endogenous tyrosine kinases are more active in these neu-

rons than in A293 cells (Fig. 3b, d). This was confirmed by biochemical analysis, which revealed that SCGs have approximately fourfold higher levels of kinase activity (data not shown). Treatment with cSRC also caused a significant time-dependent increase in the GABA-activated current after recording for 15 min (Fig. 3c, d). Overall, these observations show that neuronal GABA_A receptors can be modulated, either directly or indirectly, by tyrosine kinase activity and, in common with the observations with recombinant receptors, this seems to enhance receptor function.

To examine the likely mechanism underlying the enhancement of whole-cell GABA responses, single GABA-channel currents, activated by 10 μ M GABA, were studied in outside-out patches taken from SCG neurons in the absence and presence of internal cSRC and sodium vanadate (Fig. 4a, b). The single-channel current and chord conductances at -70 mV holding potential (control, 25.6 pS, $n=7$; +cSRC/sodium vanadate, 24.7 pS, $n=4$) were unaffected by cSRC/vanadate treatment (Table 1). However, both the mean open time and the probability of channel opening were significantly increased ($P<0.05$; Table 1). Analysis of the distribution of all open times for patches containing few simultaneous openings revealed that two exponential functions were required to fit the dwell time distribution (Fig. 4c, d). The time constants τ_{01} and τ_{02} , representing respectively the short and long open time distributions, were apparently unaffected by

FIG. 1 Tyrosine phosphorylation of GABA_A-receptor subunits expressed in A293 cells. a, Identification of receptor subunits by immunoprecipitation. Receptors were isolated from expressing cells under native (N) or denaturing (D) conditions after [³⁵S]methionine labelling with either 9E10 antisera or anti- $\alpha 1$ or $\beta 1$ antibodies, followed by SDS-PAGE (8% gels) and fluorography. Lane 1, $\gamma 2L^{(9E10)}$ alone, 9E10 (N); lane 2, $\alpha 1\beta 1\gamma 2L^{(9E10)}$, 9E10 (N); lane 3, control cells, 9E10 (N); lane 4, $\alpha 1\beta 1\gamma 2L^{(9E10)}$, anti- $\alpha 1$ (D); lane 6, $\alpha 1\beta 1\gamma 2L^{(9E10)}$, anti- $\beta 1$ (D); lanes 5 and 7, $\alpha 1\beta 1\gamma 2L^{(9E10)}$, anti- $\alpha 1$ (D) and anti- $\beta 1$ (D), respectively preadsorbed with the relevant immunogens. Numbers on the right indicate molecular weight standards. b, Western blotting of A293 total cell lysates with phosphotyrosine antibodies. Lane 1, control cells; lane 2, +100 μ M sodium vanadate for 4 h at 37 °C; lanes 3 and 5, +vSRC and receptor $\alpha 1\beta 1\gamma 2L^{(9E10)}$ subunits; lane 4, vSRC alone, blotted with a monoclonal antiserum against phosphotyrosine. 50 μ g of total protein was loaded in lanes 1-4, 250 μ g in lane 5. c, Phosphorylation of GABA_A receptors by vSRC. Expressing cells were labelled with [³²P]orthophosphoric acid and immunoprecipitated with 9E10 antisera. Receptor subunits were resolved by SDS-PAGE and autoradiography. Lane 1, vSRC alone; lane 2, $\alpha 1\beta 1\gamma 2L^{(9E10)}$ + vSRC; lane 3, $\alpha 1\beta 1\gamma 2L^{(9E10)Y(365/367)F}$ + vSRC; lane 4, $\alpha 1\beta 1\gamma 2L^{(9E10)Y(365/367)F}$ + vSRC; lane 5, $\alpha 1\beta 1\gamma 2L^{(9E10)}$ alone. d, Phosphoamino-acid analysis of GABA_A-receptor subunits. Lane 1, $\gamma 2L^{(9E10)}$ subunit (c, lane 2); lane 2, $\beta 1$ subunit (c, lane 3). The migration of phosphoserine, phosphothreonine and phosphotyrosine standards is indicated. e, Detection of wild-type and mutant forms of the $\gamma 2L^{(9E10)}$ subunit by western blotting. A293 cells expressing receptor subunits



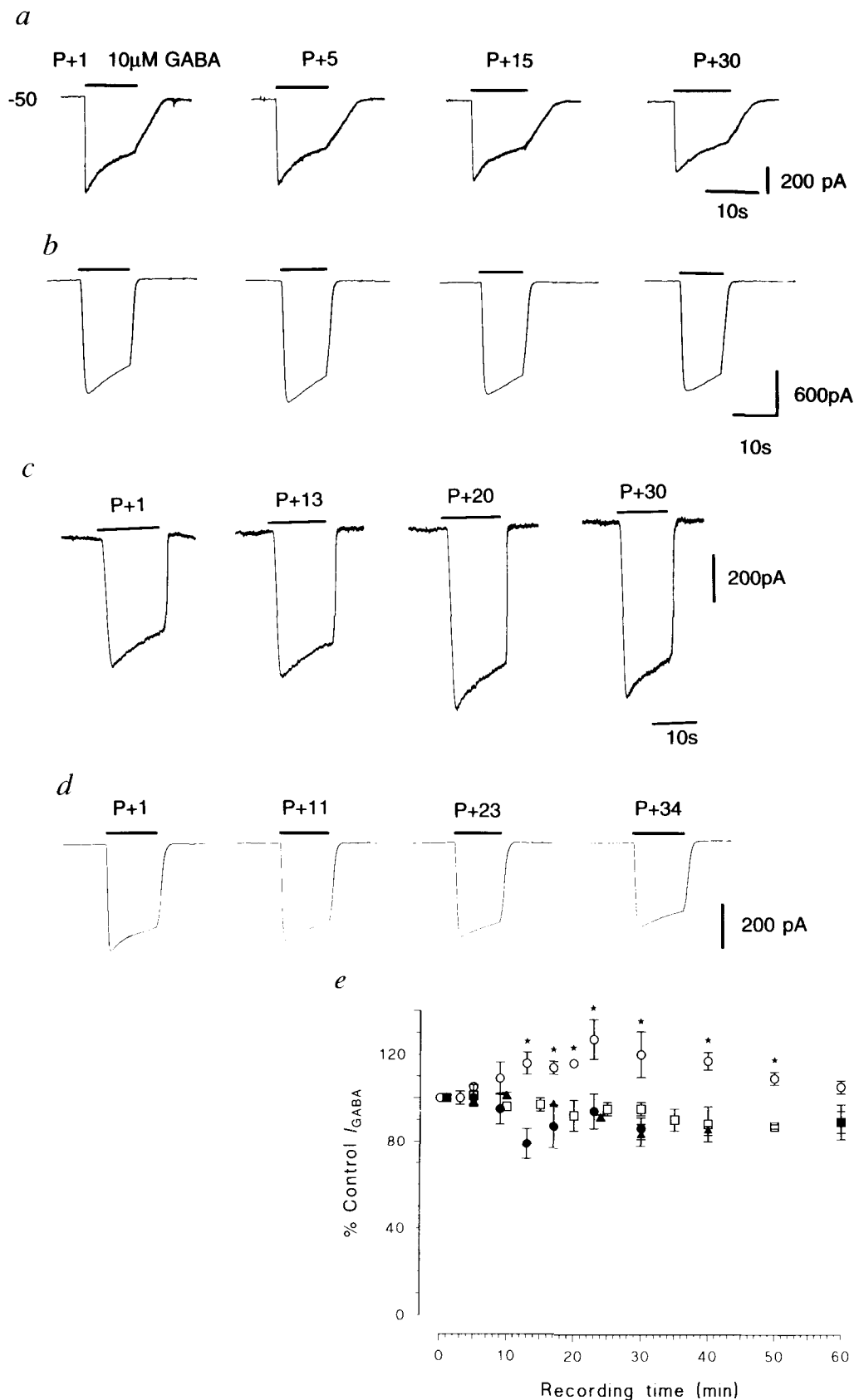
were lysed and blotted with 9E10 antisera. Lane 1, control cells; lane 2, $\alpha 1\beta 1\gamma 2L^{(9E10)}$; lane 3, $\alpha 1\beta 1\gamma 2L^{(9E10)Y(365/367)F}$.

METHODS. Expression and immunoprecipitation of GABA_A receptors in A293 cells, western blotting, phosphoamino-acid analysis and the anti- $\alpha 1$ and anti- $\beta 1$ sera have been described previously^{6-9,10,13,21,22}. The 9E10 epitope, EQKLISDEEL⁵, was added to the $\gamma 2L$ subunit between amino acids 4 and 5 by site-specific mutagenesis^{9,10}. Mutagenesis was also used to convert tyrosines 365/367 ($\gamma 2L$ subunit) and tyrosines 384/386 ($\beta 1$ subunit) to phenylalanine residues.

cSRC/vanadate (Table 1). The increased mean open time seemed to result from a shift in the relative areas of the distributions, with more long openings (22% increased to 39%) occurring at the expense of the shorter openings (78% reduced to 61%; $P < 0.05$). this effect would also increase the probability of channel opening.

In conclusion, our results suggest that tyrosine phosphorylation may be an endogenous regulatory mechanism for enhancing or maintaining GABA_A-receptor function^{19,20}. Given the ubiquity of tyrosine kinases in the central nervous system^{11,12}, and their regulation by growth factors and neurotransmitters, tyrosine phosphorylation may therefore represent a new and diverse

FIG. 2 Whole-cell responses to 10 μ M GABA recorded from A293 cells expressing receptors composed of wild-type $\alpha 1\beta 1\gamma 2$ L subunits or receptors in which biochemically identified phosphorylation sites had been mutated with or without vSRC. a, $\alpha 1\beta 1\gamma 2$ L + vSRC and b, $\alpha 1\beta 1\gamma 2$ L^{Y(365,367)F} + vSRC exposed to 100 μ M genistein by intracellular dialysis. c, d, Recordings from cells expressing only $\alpha 1\beta 1\gamma 2$ L and $\alpha 1\beta 1\gamma 2$ L^{Y(365,367)F}, respectively, exposed to cSRC (75 U ml⁻¹) and sodium vanadate (100 μ M) by intracellular dialysis. The cells were held at -50 mV and GABA was rapidly applied from a nearby Y-tube for the duration indicated by the bar. Recording times above each trace (P + x) indicate the duration in min following the attainment of the whole-cell configuration. e, Stability plot of GABA-activated membrane currents (I_{GABA}). The peak amplitudes of membrane currents activated by 10 μ M GABA in transfected A293 cells expressing either $\alpha 1\beta 1\gamma 2$ L or $\alpha 1\beta 1\gamma 2$ L^{Y(365,367)F} constructs are pooled and plotted against recording time (formation of the whole-cell recording mode is defined as $t = 0$ min). The first response to GABA, usually elicited within P + 1, was defined as 100%. Points are mean $\pm$ s.e.m. for: $\alpha 1\beta 1\gamma 2$ L, $\blacktriangle$, control pipette solution ($n = 6$ cells); $\alpha 1\beta 1\gamma 2$ L, $\bullet$, +100 μ M NaVO₃ ($n = 4$); $\alpha 1\beta 1\gamma 2$ L, $\circ$, +75 U ml⁻¹ cSRC and 100 μ M NaVO₃ ($n = 5$); $\alpha 1\beta 1\gamma 2$ L^{Y(365,367)F}, $\square$, +75 U ml⁻¹ cSRC and 100 μ M NaVO₃ ($n = 6$). * A significant difference from control untreated ($P < 0.05$ assessed by ANOVA with Bonferroni, Tukey-Kramer or Student-Newman-Keuls *post hoc* tests). Recordings were made from transiently transfected A293 cells up to 24 h after transfection with receptor expression constructs as described previously^{9,10}.



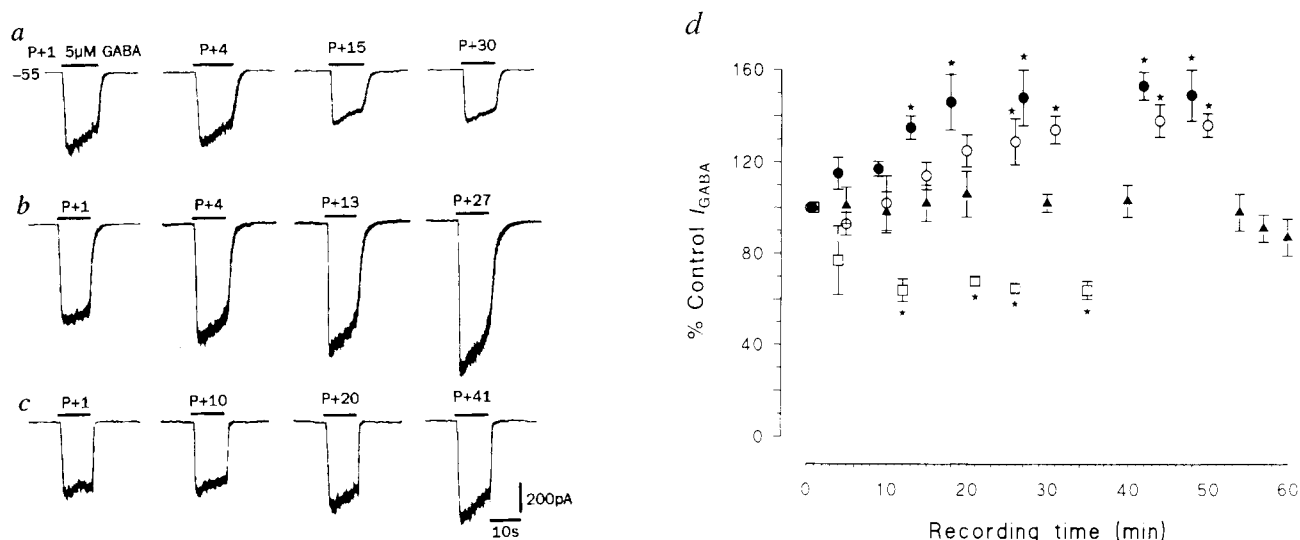


FIG. 3 Superior cervical ganglion neurons in culture were exposed to 5 μ M GABA from the Y-tube and internally perfused with 20 μ M genistein (a), 100 μ M NaVO₃ (b) or 75 U ml⁻¹ cSRC (c). Peak current amplitudes to GABA were pooled and plotted against the recording time in d. Points are mean $\pm$ s.e.m. for neurons exposed to: ●, 100 μ M NaVO₃

($n=4$); ○, 75 U ml⁻¹ cSRC ($n=3$); □, 20 μ M genistein ($n=4$); and ▲, control pipette solution ($n=7$). * Values significantly different from control ($P<0.05$ assessed by ANOVA). Superior cervical ganglia were removed from postnatal rats (P+2–4 days) and mechanically and enzymatically dissociated as described previously^{9,10,18}.

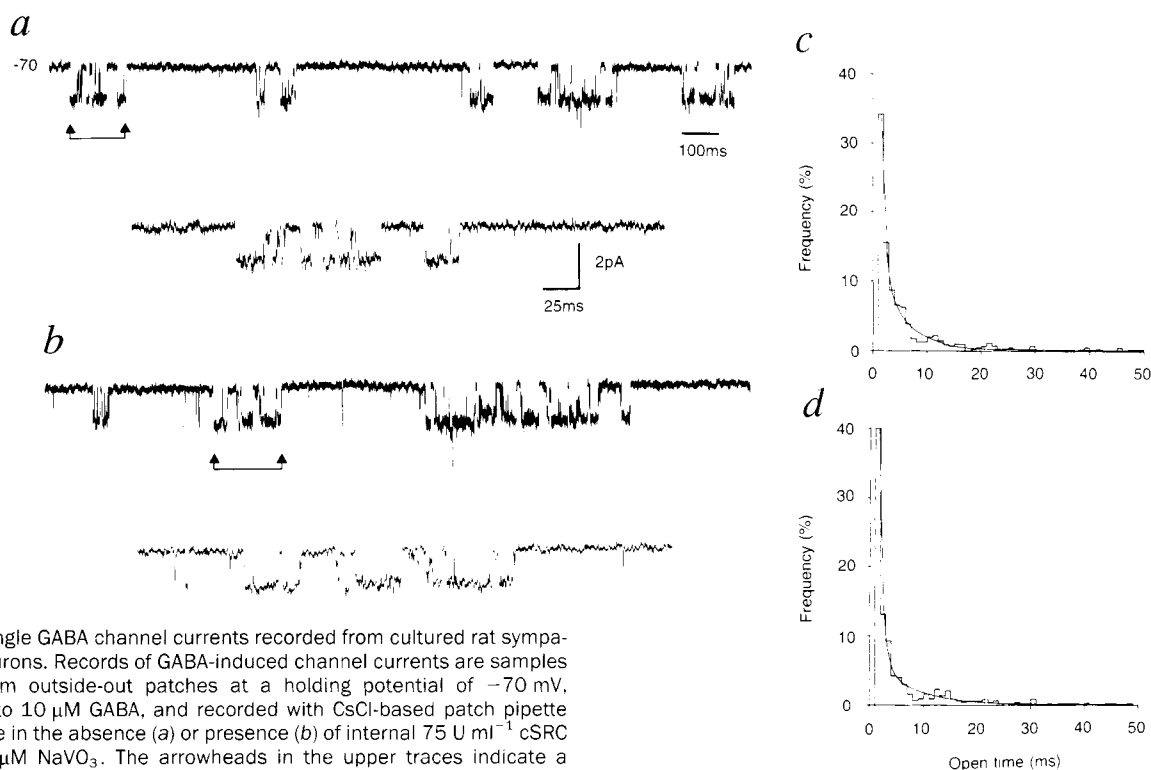


FIG. 4 Single GABA channel currents recorded from cultured rat sympathetic neurons. Records of GABA-induced channel currents are samples taken from outside-out patches at a holding potential of -70 mV, exposed to 10 μ M GABA, and recorded with CsCl-based patch pipette electrolyte in the absence (a) or presence (b) of internal 75 U ml⁻¹ cSRC and 100 μ M NaVO₃. The arrowheads in the upper traces indicate a portion of each record that has been selected for expansion at a higher time resolution and is shown in the lower traces. In the absence (c) or presence (d) of cSRC and NaVO₃ the mean open time distributions, for all openings longer than 100 μ s, were described by the sum of two exponential components.

METHODS. Outside-out patches were obtained from cultured rat sympathetic neurons bathed in control Krebs solution as described previously¹⁸. To induce GABA-activated single-channel currents, the excised patch was positioned juxtaposed to a Y-tube from which 10 μ M GABA was rapidly applied. The single-channel currents were resolved using an EPC7 amplifier and stored on a Racal tape recorder (d.c. to 5 kHz). Currents were filtered at 1–3 kHz (-3dB; 6-pole Bessel) and digitized at 5–15 kHz before analysis using software provided by J. Dempster (PAT ver. 7; University of Strathclyde). The measurements of

open durations were made using a 50% threshold cursor to the main conductance state of 25 pS. The transition detection of open and closed states was used to form an idealized record from which individual open durations were measured and collated into the frequency distributions. The distributions were fitted with the sum of exponential functions using a nonlinear least squares, Levenberg-Marquadt fitting routine as described previously¹⁸. Single-channel currents were accepted for kinetic analysis if there was apparently only one channel active, or when the number of multiple channel openings never exceeded 2% of the total number of open events¹⁸.

process for modulating inhibition mediated via GABA_A receptors and consequently cell excitability.

Note added in proof: Similar modulation of GABA_A receptor function has recently been reported elsewhere²³. □

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Induction of apoptosis in mature T cells by tumour necrosis factor

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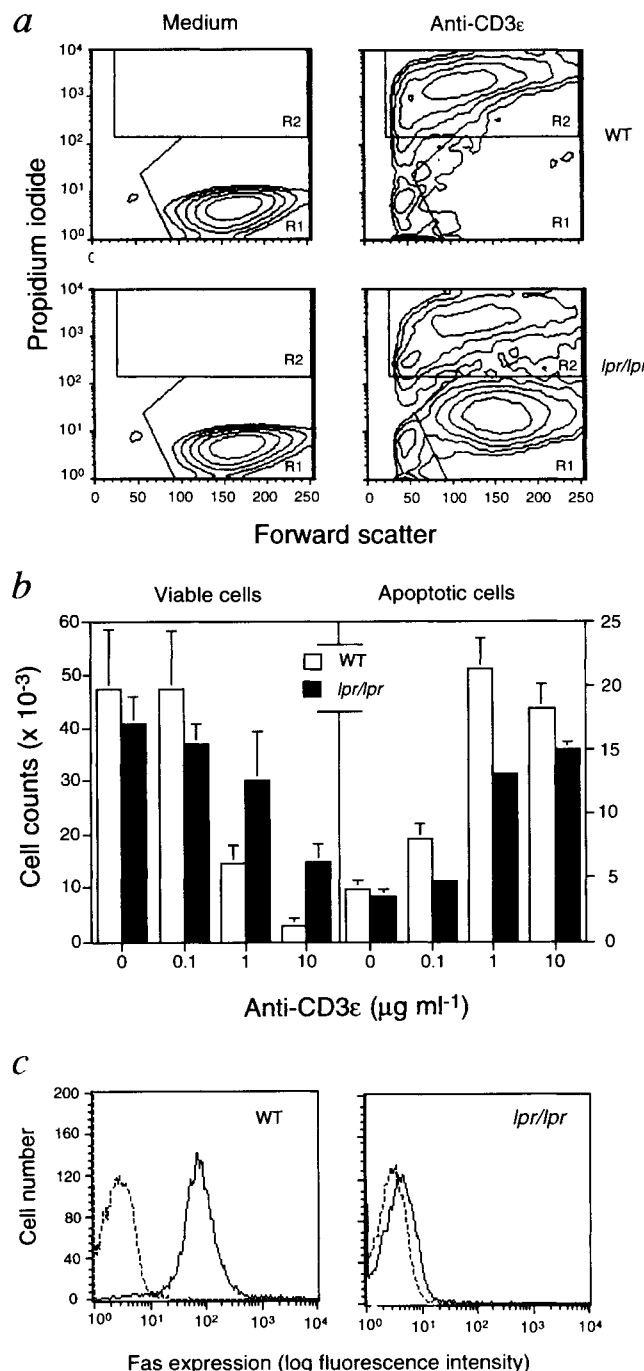
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T-CELL receptor-induced apoptosis regulates immune responses and can result from interactions between Fas (Apo1/CD95) and Fas ligand (FasL)^{1–12}. Mutations in the genes for Fas and FasL cause disorders resembling human autoimmune diseases in *lpr* and *gld* mice, respectively^{13,14}. However, peripheral T-cell deletion takes place in *lpr* mice, and autoimmune syndromes occur in mouse strains without Fas or FasL defects^{15,16}. Here we show that tumour necrosis factor (TNF) can mediate mature T-cell receptor-induced apoptosis through the p75 TNF receptor. Blockage of both TNF and FasL is required to abrogate T-cell death and TNF mediates the death of most CD8⁺ T cells, whereas FasL mediates the death of most CD4⁺ T cells. Our results suggest that autoregulatory apoptosis of the mature T cells can occur by two distinct molecular mechanisms.

Resting lymph-node T cells (LNTC) from C3H/HeJ wild-type, *lpr* (Fas-deficient), or *gld* (FasL-deficient) mice were primed for proapoptotic apoptosis by concanavalin A and interleukin (IL)-2 treatment^{4,5}. Dramatic cell death in wild-type LNTC was caused by crosslinking of the T-cell antigen receptor (TCR)/CD3 complex (Fig. 1a). We also found that *lpr* T cells, although less sensitive, manifested substantial apoptosis with increasing TCR stimulation (Fig. 1a, b). Death of *lpr* T cells did

not seem to be due to 'leakiness' of the Fas mutation as *lpr* T cells had almost no surface expression of Fas compared to wild-type T cells (Fig. 1c)^{13,17}. We therefore tested whether TNF, an apoptosis-inducing cytokine that shares amino acid homology with FasL, caused *lpr* T-cell death¹⁸. Under conditions in which TCR stimulation causes the death of IL-2 treated T cells, TNF messenger RNA and protein were strongly induced (Fig. 2a). We then tested the contributions of FasL and TNF to apoptosis by blocking with a Fas-immunoglobulin Fc fusion protein (Fas-Fc)¹⁹ and an anti-TNF- α serum (anti-TNF)²⁰, respectively. After 48 hours of TCR stimulation, Fas-Fc slightly reduced apoptosis in wild-type LNTC but not in *lpr* LNTC, whereas anti-TNF partly rescued wild-type cells but completely abrogated *lpr* T-cell death (viable cells were increased) (Fig. 2b). Fas-Fc and anti-TNF together blocked all cell loss in both wild-type and *lpr* T cells. Anti-TNF also completely prevented apoptosis in LNTC



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FIG. 2 Fas-independent T-cell apoptosis is mediated by TNF. **a**, The left panel shows a northern blot of TNF, FasL and actin mRNAs either before (1) or 8 h after anti-CD3 ϵ stimulation (2), and the right panel shows the 24 h accumulation of TNF bioactivity as measured by an ELISA assay (Genzyme). **b**, Viable cell loss of C3H wild-type, *lpr* or *gld* T cells after 48 h stimulation with 5 $\mu\text{g ml}^{-1}$ anti-CD3 ϵ in the presence of Fas-Fc or anti-TNF- α as indicated. Negative cell loss indicates enhanced proliferation or survival (percentage increase over control cell recovery). Fas-Fc and anti-TNF do not induce apoptosis, and TNF blockade causes a 10–30% increase in unstimulated T-cell viability. **c**, Time course of viable and apoptotic cell accumulation after stimulation with 10 $\mu\text{g ml}^{-1}$ plate-bound anti-CD3 ϵ and various additions: medium only (white bars), 5 $\mu\text{g ml}^{-1}$ TNFR-Fc (hatched bars), 5 $\mu\text{g ml}^{-1}$ Fas-Fc (grey bars) and 5 $\mu\text{g ml}^{-1}$ TNFR-Fc and 5 $\mu\text{g ml}^{-1}$ Fas-Fc (black bars). **METHODS**. IL-2-treated LNTC were prepared, stimulated and quantified for viable cells as in Fig. 1. Cultures contained 5 $\mu\text{g ml}^{-1}$ Fas-Fc, 5 $\mu\text{g ml}^{-1}$ TNFR-Fc, or 1:50 dilutions of anti-TNF antiserum (Genzyme) as indicated. Northern blots were performed using FasL and TNF probes as previously described¹¹. ELISA was performed according to the manufacturer's instructions. Values are averages of two independent determinations using pools from two microtitre wells.

from *gld* mice (Fig. 2b). Apoptosis was not prevented by control serum, a CD30-Fc fusion protein or a lymphotoxin- β receptor-Fc fusion protein (data not shown). TNF could therefore account for all TCR-induced death in *lpr* or *gld* T cells and for significant wild-type T-cell death.

FasL- and TNF-mediated T-cell apoptosis could be distinguished kinetically (Fig. 2c). The apoptosis observed at 24 hours was inhibited by Fas-Fc but not by TNFR-Fc. At 48 hours, overall apoptosis was greater and now could be decreased by either TNFR-Fc or Fas-Fc, and almost completely by both blocking agents together. These findings suggest that T-cell death at 24 hours was mostly due to FasL, whereas death at 48 hours had both FasL and TNF components. Interestingly, protection could be obtained with single blocking agents (either Fas-Fc or TNFR-Fc alone), suggesting that many T cells may be sensitive only to FasL or TNF but not both. Viable cell recovery increased when FasL or TNF were blocked, indicating that T cells continue to proliferate when apoptosis is prevented (Fig. 2c).

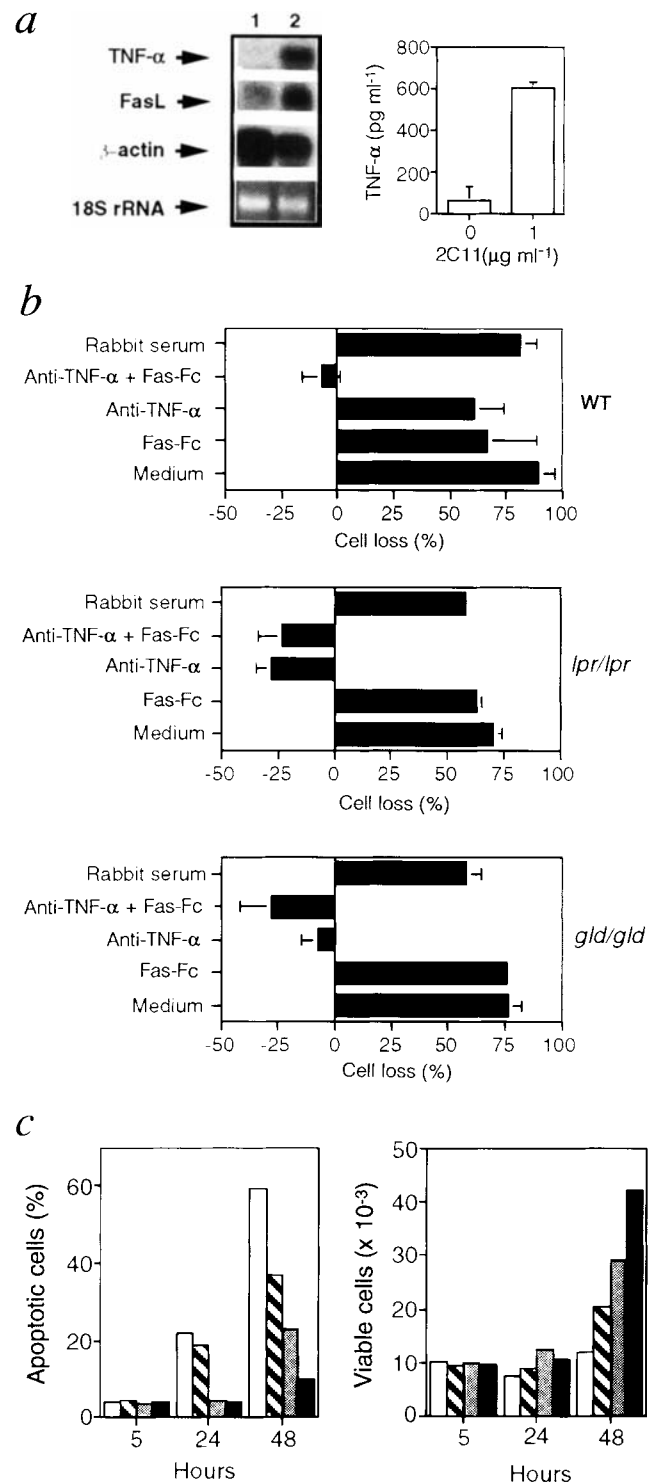
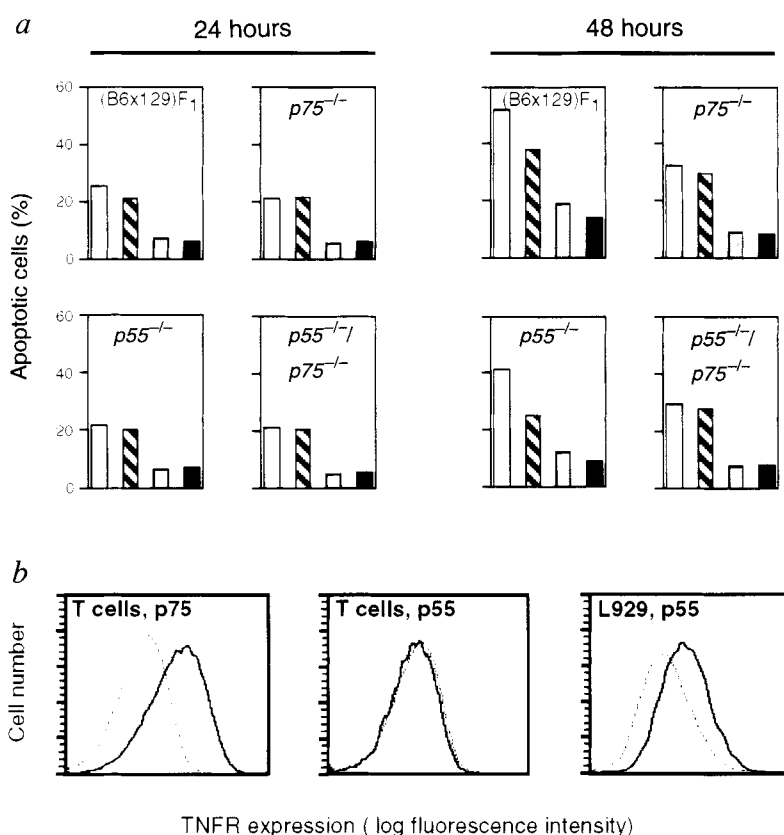


FIG. 1 T lymphocytes from *lpr* mice undergo apoptosis after TCR/CD3 crosslinking. **a**, Flow cytometric plots displaying propidium iodide (PI) fluorescence versus forward light scatter for C3H wild-type (WT) and C3H.*lpr* T cells after 48 h with IL-2 in either uncoated wells or wells coated with 10 $\mu\text{g ml}^{-1}$ anti-CD3 ϵ . Viable cells, which have a high forward scatter (large size) and low PI fluorescence, are in the R1 gate. Dead cells, which have low forward scatter and high PI fluorescence, are in the R2 gate. Cell counts for untreated cells were: for wild-type, R1, 66,686, and R2, 3,613; for *lpr/lpr*, R1, 66,722, and R2, 3,308. For anti-CD3 ϵ -treated cells they were: wild-type, R1, 1,363, and R2, 19,780; for *lpr/lpr*, R1, 20,327, and R2, 15,430. **b**, The viable cell loss and percentage increase in apoptotic cells (y axes) for wild-type (white bars) and *lpr/lpr* (black bars) T cells after 48 h with platebound anti-CD3 ϵ as indicated. Values are arithmetic means from duplicate pools of 3 wells each. The percentage deletion of viable cells for 0.1, 1 and 10 $\mu\text{g ml}^{-1}$ 2C11 was 16.6, 95.2 and 97.9, respectively, in wild-type cells and was 9.4, 47.9 and 71.2, respectively, in *lpr* cells. A time course showed that deletion in *lpr* and *gld* T cells was most evident at 48 and 72 h (data not shown). **c**, Fas expression using phycoerythrin (PE) fluorescence, on IL-2-treated LNTCs from wild-type and *lpr/lpr* C3H mice. The broken line indicates cells that were not stained, and the solid line indicates cells stained with PE-labelled hamster anti-Fas (Jo2) antibody. **METHODS**. C3H/HeJ wild-type, C3H.MRL.*lpr*, or C3H.*gld* mice (Jackson Laboratories, Bar Harbor, ME) were 6 weeks old, which is before the onset of lymphoproliferation. LNTCs treated with 5 $\mu\text{g ml}^{-1}$ concanavalin A for 48 h, washed with 10 mg ml^{-1} α -methylmannoside, and incubated in 50 IU ml $^{-1}$ IL-2 to predispose to apoptosis as previously described^{4,5}. Cells (50,000) ($>98\%$ $\alpha\beta$ T cells) were then stimulated with platebound mAb 145-2C11 (anti-CD3 ϵ). Viable cells were quantified for 30 s at a constant rate using a Becton-Dickinson FACSCAN with CELLQUEST software⁵. Data are representative of 8 experiments.

TNF exerts its biological effect by binding to one of two TNF receptors (TNFR): either p55, which has an intracytoplasmic, signalling 'death domain' homologous to Fas, or p75, which signals by means of a different intracytoplasmic domain^{18,21,22}. We assessed the role of either TNFR in T-cell death by generating mice with homozygous deficiencies in p55 (*2p55* $^{-/-}$), p75 (*p75* $^{-/-}$), or both (*p55* $^{-/-}$, *p75* $^{-/-}$) (Fig. 3)²³. In all strains, death at 24 hours was blocked only by Fas-Fc, confirming the more rapid action of Fas. At 48 hours, apoptosis in (B6x129)F $_1$ or *p55* $^{-/-}$ T cells was decreased by either TNFR-Fc or Fas-Fc alone and reduced to background levels by adding both (Fig. 3a). In T cells from *p75* $^{-/-}$ or double-deficient (*p55* $^{-/-}$, *p75* $^{-/-}$) mice, apoptosis was decreased by Fas-Fc but not by TNFR-Fc

FIG. 3 p75 TNF receptor involvement in T-cell apoptosis. **a**, Apoptosis at 24 h and 48 h after TCR stimulation with T cells from various mouse strains as indicated. Apoptotic cell accumulation after stimulation with $10 \mu\text{g ml}^{-1}$ platebound anti-CD3 ϵ and various additions as indicated in the Fig. 2c legend. **b**, Activated T cells from wild-type C57BL/6 mice or control L929 fibroblast cells stained for TNFRs. Unstained cells (thin line) are compared with fluorescence obtained after staining with either the anti-p55 mAb (p55R176) or the p75 mAb (TR75-45) (thick line²⁹). T cells were not stained by 2 other anti-p55 TNFR mAbs, p55R286 and p55R593 (ref. 29), and direct addition to TNF to p55 $^{-/-}$ T cells caused apoptosis. (data not shown). **METHODS**. Mice with homozygous deficiencies in the p55 and p75 TNFRs were prepared by targeting the genes in embryonic stem (ES) cells to create null alleles of the TNFR genes by replacement with a neomycin resistance gene using the positive and negative selection procedure as described previously²³. ES cells targeted at either the p55 TNFR or the p75 TNFR locus were used to generate chimaeric mice and for transmission and breeding to obtain mice bearing homozygous deficiencies in the gene for either TNFR p55 or TNFR p75. These mice were then used for intercross breeding to obtain double-deficient (p55 $^{-/-}$ /p75 $^{-/-}$) mice. Control experiments verified that there was no expression of wild-type mRNA or protein from the targeted loci in these mice (data not shown). Details of these strains will be reported elsewhere. Activated T cells from the indicated mouse strains were exposed to $10 \mu\text{g ml}^{-1}$ platebound anti-CD3 ϵ (500.A2, Pharmingen) for various times as indicated. The apoptotic cell fraction was quantified using PI and Hoechst 33342 in combination with forward scatter multiparameter flow cytometry with an Epics Elite cytometer (Coulter, Hialeah, FL) equipped with argon (488-nm emission) and helium-cadmium (325-nm emission) lasers⁶. Values are from pools of 12 individual replicate wells and are representative of 3 independent experiments. T cells or L929 cells were stained with anti-TNFR mAbs²⁹ for 60 min,

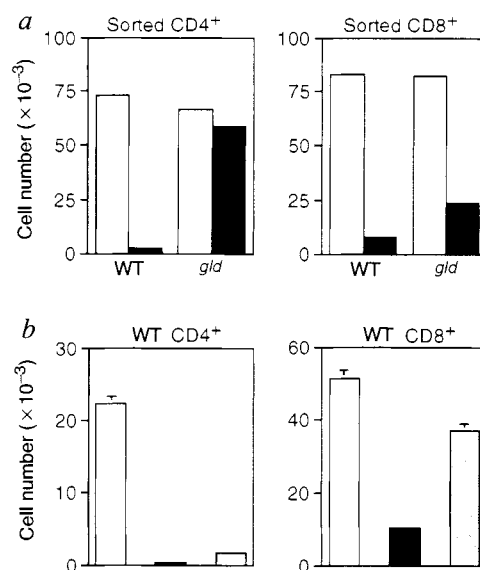


washed and developed with a fluorescein-conjugated goat anti-hamster antibody (Pharmingen) and analysed on a Becton-Dickinson FACSCAN using Lysis II software; graphs display 10,000 events.

(Fig. 3a). Although the strains contained variable contributions of the C57BL/6 and 129 backgrounds, T cells from p75 $^{-/-}$ or double-deficient mice reproducibly underwent less apoptosis at 48 hours compared to those from p55 $^{-/-}$ or C57BL/6 mice. This suggested that there was little or no TNF-mediated apoptosis in T cells without the p75 TNFR. Furthermore, flow cytometry

showed that wild-type T cells were stained by a monoclonal antibody specific for p75, but not by p55-specific monoclonal antibodies, although control L929 fibroblasts expressed p55 detectable by the same monoclonal antibody (Fig. 3b). Substantial binding of TNF could be demonstrated to wild-type and p55 $^{-/-}$ T cells, but not to p75 $^{-/-}$ or double-deficient cells (data

FIG. 4 FasL and TNF primarily mediate the apoptosis of CD4 $^{+}$ and CD8 $^{+}$ T cells, respectively. **a**, Comparison of the susceptibility of magnetic bead sorted populations of CD4 $^{+}$ and CD8 $^{+}$ T cells from either wild-type (+/+) or *gld/gld* T cells to TCR-stimulated apoptosis. Histograms show cell number from samples either on uncoated plates (white bars) or plates coated with $10 \mu\text{g ml}^{-1}$ anti-CD3 ϵ (black bars). **b**, The recovery of IL-2-stimulated CD4 $^{+}$ and CD8 $^{+}$ T cells from wild-type (WT) LNTCs after anti-CD3 ϵ stimulation with or without 1:150 anti-TNF serum. Histograms show the number of viable CD4 $^{+}$ and CD8 $^{+}$ T cells recovered from unstimulated controls (white bars), cells stimulated with anti-CD3 ϵ (black bars) or cells stimulated with anti-CD3 ϵ with anti-TNF (grey bars). Values are means from 4 samples. **METHODS**. CD4 $^{+}$ and CD8 $^{+}$ T cells were separated by staining with either fluorescein anti-CD4 or anti-CD8 mAbs (Pharmingen), binding to anti-fluorescein magnetic particles, and then separating with an external magnetic field (BioMag, Perseptive Diagnostics). Populations were >98% pure. Separated CD4 $^{+}$ or CD8 $^{+}$ cells (50,000) were incubated for 48 h in wells that were either uncoated or coated with 1 or $10 \mu\text{g ml}^{-1}$ anti-CD3 and then analysed by flow cytometry as described in the legend to Fig. 1. The same protocol was followed for unseparated T-cell populations, but then the cells were stained with phycoerythrin-conjugated anti-CD4 antibody and fluorescein-conjugated anti-CD8 antibody (Pharmingen), analysed on a Becton-Dickinson FACSCAN using CELLQUEST software and the viable cells were quantified after gating for either CD4 or CD8.



not shown). Taken together these results suggest that p75 is the principle TNFR on T lymphocytes and is sufficient for TNF-mediated T-cell apoptosis.

Finally, we assessed the role of TNF and Fas-mediated apoptosis in the CD4 and CD8 T-cell subsets. We found that the *gld* mutation in FasL almost completely blocked TCR-induced apoptosis of sorted CD4⁺ T cells but was incapable of preventing apoptosis of most CD8⁺ T cells (Fig. 4a). By contrast, anti-TNF hardly protected CD4⁺ T cells, but prevented TCR-induced death of most CD8⁺ T cells (Fig. 4b). Similar results were obtained with *lpr* T cells (data not shown).

We have found that TNF mediates Fas-independent mature T-cell apoptosis and may account for peripheral deletion in *lpr* mice^{10,15,24}. In contrast to mature T cells, blocking Fas and TNF had no effect on thymocyte death *in vitro* (data not shown).

TNF caused death at later times than Fas and was transduced by p75. This suggests a physiological role for p75 which does not contain homology to the Fas 'death domain' and uses different signalling pathways from the p55 TNFR that mediates apoptosis of non-lymphoid cells^{19,21,25,26}. We also found that Fas alone accounted for almost all CD4⁺ T-cell death, whereas TNF caused most CD8⁺ T-cell death. CD8⁺ T cells may therefore use FasL primarily to kill target cells and may rely on the slower TNF pathway for autoregulatory apoptosis. Our findings may explain why Fas defects in mice and humans cause humorally mediated autoimmune disorders^{27,28} and why the virally induced deletion of CD8⁺ T cells occurs in *lpr* mice²⁴. It will be important to determine how these two distinct molecular pathways of apoptosis mediate mature T-cell homeostasis in other autoimmune and infectious diseases. □

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Facilitation of *lin-12*-mediated signalling by *sel-12*, a *Caenorhabditis elegans* S182 Alzheimer's disease gene

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THE *lin-12* and *glp-1* genes of *Caenorhabditis elegans* are members of the *lin-12/Notch* family of receptors for intercellular signals that specify cell fate^{1,2}. By screening for suppressors of a *lin-12* gain-of-function mutation, we identified a new gene, *sel-12*, which appears to function in receiving cells to facilitate signalling mediated by *lin-12* and *glp-1*. The *sel-12* gene encodes a protein with multiple transmembrane domains, and is similar to S182, which has been implicated in early-onset familial Alzheimer's disease³. The high degree of sequence conservation suggests that the function of the SEL-12 and S182 proteins may also be conserved.

The *lin-12(d)* hypermorphic mutation *lin-12(n950)* causes a Multivulva phenotype characterized by the production of ectopic pseudovulvae^{4,5}. We screened for non-Multivulva revertants after ethyl methanesulphonate mutagenesis⁵ of *lin-12(n950)* hermaphrodites; two recessive suppressors, *ar131* and *ar133*, proved to be alleles of a new gene, *sel-12* (*sel* means suppressors

and/or enhancer of *lin-12*). These *sel-12* alleles cause an incompletely penetrant, recessive egg-laying-defective (Egl) phenotype in a *lin-12(+)* background. Because *sel-12(ar131)* is viable, fertile and Egl *in trans* to a deficiency (data not shown), we also performed a screen for mutations that fail to complement the Egl defect of *sel-12(ar131)*. From a screen of 5,900 mutagenized haploid genomes we identified two additional *sel-12* alleles. One allele obtained in this screen, *sel-12(ar171)*, displays a completely penetrant Egl defect as a homozygote and *in trans* to a deficiency, suggesting that *sel-12(ar171)* strongly reduces *sel-12* function. This inference is supported by the molecular analysis described below, which indicated that the *ar171* lesion would result in a truncated protein product.

The Egl phenotype caused by *sel-12* mutations in a *lin-12(+)* background is reminiscent of the Egl phenotype caused by reducing *lin-12* activity (see Table 1 legend). However, a more general involvement of *sel-12* in *lin-12*- and *glp-1*-mediated cell-fate decisions becomes apparent when the phenotypes of *lin-12*; *sel-12* and *glp-1*; *sel-12* double mutants are analysed (Table 1). We examined the genetic interactions of *sel-12* with two *lin-12* hypomorphic mutations, with a *lin-12(d)* hypermorphic mutation, and with a *glp-1* hypomorphic mutation. In all cases we found that reducing *sel-12* activity reduces *lin-12* or *glp-1* activity. These genetic interactions are exemplified by the effects of *sel-12* on two *lin-12*-mediated decisions, the anchor cell, ventral uterine precursor cell (AC/VU) decision and vulval precursor cell (VPC) specification.

The AC/VU decision involves an interaction between two initially equivalent cells of the somatic gonad, Z1.ppp and Z4.aaa. In a given hermaphrodite, Z1.ppp and Z4.aaa interact so that one of these cells becomes the AC and the other a VU^{7,9}. When *lin-12* activity is eliminated, both Z1.ppp and Z4.aaa become ACs (the '2 AC defect'), and when *lin-12* is activated, as in *lin-12(d)* mutants, both Z1.ppp and Z4.aaa become VUs

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the cDNA clone after generating systematic deletions using the Erase-a-base system (Promega). DNA sequencing was performed on double-stranded templates using Sequenase (US Biochemical). The cDNA contained both a poly(A) tail and a portion of the spliced leader sequence SL1 (ref. 29), suggesting it was a full-length clone. We confirmed the 5' end of the cDNA by reverse transcription polymerase chain reaction (RT-PCR)³⁰. The sequence of this full-length cDNA can be found through GenBank under accession number U35660. To identify the lesions associated with *sel-12* alleles we used PCR to amplify the *sel-12* genomic fragment from DNA isolated from the *sel-12* mutant strains using the primers DL103 (5'-TGTCTGAGTTACTAGTTTCC-3') and DLG3 (5'-GGAATCTGAAGCACCTGTAAGCAT-3'). A portion of this double-stranded amplification product was used as the template in a subsequent round of PCR using only the primer DL103, to generate a single-stranded template. Exon-specific primers were used to determine the entire coding sequence for all three alleles. For each allele, only one alteration in sequence was identified.

TABLE 1 *sel-12(ar171)* reduces *lin-12* and *glp-1* activity

(a) Enhancement of hypomorphic <i>lin-12</i> alleles by <i>sel-12(ar171)</i>				
Genotype	% 2 ACs	% Ventral coelomocytes	Fertility	% L1 arrest*
Wild-type <i>C. elegans</i> var. Bristol strain N2	0	0	Yes	0
<i>sel-12(ar171)unc-1(e538)</i>	0 (<i>n</i> = 108)	0 (0/17)	Yes	0 (<i>n</i> = 233)
<i>lin-12(n676n930); unc-1(e538)</i>	30 [†]	8 (1/12)	Yes	9 (<i>n</i> = 233)
<i>lin-12(n676n930); sel-12(ar171)unc-1(e538)</i>	95 (<i>n</i> = 41)	92 (12/13)	No	17 (<i>n</i> = 177)
<i>lin-12(ar170); unc-1(e538)</i>	16 (<i>n</i> = 32)	0 (0/32)	Yes	0 (<i>n</i> = 209) [‡]
<i>lin-12(ar170); sel-12(ar171)unc-1(e538)</i>	98 (<i>n</i> = 47)	0 (0/47)	Yes	0 (<i>n</i> = 111)
<i>lin-12(0)</i>	100 [§]	100 [§]	No	10

(b) Suppression of a hypermorphic <i>lin-12</i> allele by <i>sel-12(ar171)</i>		
Genotype	Number of VPCs adopting a vulval fate/hermaphrodite	% 0 AC
Wild-type <i>C. elegans</i> var. Bristol strain N2	3	0
<i>lin-12(n950); unc-1(e538)</i>	6 (<i>n</i> = 7)	100
<i>sel-12(ar171)unc-1(e538)</i>	3 (<i>n</i> = 10)	0 (<i>n</i> = 10 8)
<i>lin-12(n950); sel-12(ar171)unc-1(e538)</i>	2–4 (<i>n</i> = 8)	89.5 (<i>n</i> = 5 7)

(c) Enhancement of <i>glp-1(e2141)</i> by <i>sel-12(ar171)</i>		
Genotype	% Sterility in both gonad arms	% Sterility in one gonad arm
Wild-type <i>C. elegans</i> var. Bristol strain N2	0	0
<i>glp-1(e2141); unc-1(e538)</i>	8.5 (<i>n</i> = 259)	4.0 (<i>n</i> = 259)
<i>sel-12(ar171)unc-1(e538)</i>	0	0
<i>glp-1(e2141); sel-12(ar170)unc-1(e538)</i>	25 (<i>n</i> = 422)	8.8 (<i>n</i> = 422)

Most *lin-12*- and *glp-1*-mediated cell fate decisions appear normal in *sel-12(ar171)* mutants. However, the egg-laying defect of *sel-12(ar171)* hermaphrodites resembles the egg-laying defect of *lin-12* hypomorphic mutants¹¹: *sel-12(ar131)* hermaphrodites leak occasional eggs and larvae, and like *lin-12* hypomorphic mutants, *sel-12* mutants have morphologically normal hermaphrodite-specific neuron (HSNs), sex muscles and VPC lineages. Egg laying is particularly sensitive to reduction in *lin-12* activity (ref. 11 and H. Wilkinson and I.G., unpublished observations). It is therefore possible that both *lin-12* and *sel-12* are required for an as yet unidentified cell fate decision(s) underlying the egg-laying defect. That *sel-12(ar171)* mutants do not display all of the defects associated with loss of *lin-12* function may indicate that *sel-12(ar171)* is not a null allele or *sel-12* function is partly redundant with the function of another gene. (a) Cell fate transformations were scored at 25 °C using criteria described in ref. 4 unless otherwise indicated. At 25 °C *lin-12(n676n930)* behaves like a hypomorph, whereas at 15 °C *lin-12(n676n930)* has mildly elevated *lin-12* activity¹¹. Because *lin-12(n676n930)*; *sel-12(ar171)* hermaphrodites are sterile at 25 °C, we shifted fertile *lin-12(n676n930)*; *sel-12(ar171)* hermaphrodites from 15 to 25 °C so that their progeny could be scored for cell fate transformations and other defects. *lin-12(ar170)* behaves like a hypomorph for the AC/VU decision (J. Hubbard and I.G., unpublished observations). In strains containing *lin-12(ar170)*, cell fate transformations were scored in hermaphrodites raised at 20 °C; other defects were scored in the progeny of hermaphrodites grown at 20 °C and shifted to 25 °C. 2 ACs (%): in *lin-12(0)* mutants, both Z1.ppp and Z4.aaa become ACs, so *lin-12(0)* hermaphrodites have two ACs; in *lin-12(d)* mutants such as *lin-12(n950)*, both Z1.ppp and Z4.aaa become VUs, so *lin-12(d)* hermaphrodites have 0 ACs. The number of anchor cells was scored in the L3 stage using Nomarski microscopy. For all genotypes, hermaphrodites either had one or two ACs. Ventral coelomocytes: the fates of two pairs of cells, M.d(l)/rpa and M.v(l)/rpa are affected by mutations in *lin-12*. In wild type, the ventral pair of cells gives rise to one sex myoblast and one body muscle; the dorsal pair gives rise to coelomocytes. In *lin-12(0)* animals, the ventral pair as well as the dorsal pair gives rise to coelomocytes, so that *lin-12(0)* hermaphrodites have extra ventral coelomocytes; in *lin-12(d)* animals, both pairs of cells give rise to sex myoblasts/body muscles. The presence of ventral coelomocytes was scored in the L3 stage. For all genotypes, the absence of ventral coelomocytes suggests that the sex myoblast was specified normally (see ref. 4). Fertility: fertility was scored by the appearance of eggs either on the plate or inside the hermaphrodite and the ability to propagate the strain. L1 arrest: full viability requires activity of *lin-12* or a related gene, *glp-1*. *lin-12(0)* *glp-1(0)* double mutants display a fully penetrant L1 arrest phenotype and a Lag phenotype characterized by specific cell fate transformations²³. *lin-12(0)* single mutants display a low penetrance L1 arrest phenotype and a somewhat lower penetrance Lag phenotype²³. Single gravid hermaphrodites were placed on a plate at 25 °C. Most of the hermaphrodites were completely egg-laying defective and laid no eggs; some *lin-12(n676n930)* animals released a few eggs or larvae before turning into 'bags of worms', in which case the hermaphrodite was transferred after a day. Because *lin-12(n676n930)* animals can grow slowly at 25 °C, L1 arrested animals were scored for 3 days after all the eggs had hatched. Arrested L1 animals were spotchecked for the presence of Lag phenotypes using Nomarski microscopy. Some arrested L1 animals of each genotype displayed Lag phenotypes (data not shown). (b) Animals were grown at 20 °C. VPC fates were scored by determining the cell lineages of P3.p–P8.p in each animal (Table 2 and data not shown). The number of ACs were scored as described above. For all genotypes, hermaphrodites had either 0 or 1 AC. (c) *glp-1(e2141ts)* is weakly hypomorphic at 20 °C and essentially wild type at 15 °C (ref. 24). Strains containing *glp-1(e2141)* were maintained at 15 °C; fertile adults grown at 15 °C were placed at 20 °C, and their progeny grown at 20 °C were scored for sterility. Other strains were maintained continuously at 20 °C. *glp-1* activity controls the decision of germline nuclei between mitosis and meiosis (refs 24, 25 and L. W. Berry and T. Schedl, personal communication). GLP-1 is thought to be the receptor for the inductive signal from the distal tip cells of the somatic gonad that promotes germline mitosis (and/or inhibits meiosis)⁷. When *glp-1* activity is eliminated, germline nuclei enter meiosis²⁵. Hermaphrodites of each genotype were scored for sterility in one or both gonad arms in the dissecting microscope. Several sterile or half-sterile individuals were examined by Nomarski microscopy, and sterile gonad arms were found to have the characteristic Glp phenotype (data not shown).

* Some L1-arrested animals were examined for Lag phenotypes: lack of an anus and rectum, lack of an excretory cell, and a twisted nose. Those phenotypes were observed for all genotypes where L1-arrested animals were identified.

[†] See ref. 11.

[‡] *lin-12(ar170)* (not *unc-1*).

[§] *lin-12(n137n720)*; see ref. 4.

|| *lin-12(n941)*; see ref. 23.

(the '0 AC defect')^{4,10}. Two observations indicate that *sel-12* reduces *lin-12* activity in Z1.ppp and Z4.aaa. First, *sel-12* dramatically enhances the penetrance of the 2 AC defect of *lin-12* hypomorphs (Table 1a). For example, 30% of *lin-12(n676n930)* hermaphrodites have 2 AC¹¹, whereas essentially all *lin-12(n676n930); sel-12(ar171)* have 2 ACs. Second, *sel-12* partly suppresses the 0 AC defect caused by LIN-12 activation (Table 1b). For example, all *lin-12(n950)* hermaphrodites lack an AC, whereas 10% of *lin-12(n950); sel-12(ar171)* hermaphrodites have an AC.

Each of the six VPCs, P3.p–P8.p, has the potential to adopt one of two vulval fates, termed primary (1°) and secondary (2°)

or a non-vulval fate, termed tertiary (3°) (refs 12, 13). Normally, P5.p, P6.p and P7.p adopt vulval fates, in a 2°–1°–2° pattern¹⁴. This pattern is the outcome of the integration of two signalling inputs: a *let-60* Ras-mediated inductive signal from the AC induces vulval fates, and a *lin-12*-mediated lateral signal between VPCs prevents adjacent VPCs from adopting the 1° fate (reviewed in ref. 15). The *let-60* Ras-mediated inductive signal may cause expression or activation of the lateral signal^{16,17}, which activates LIN-12 to cause a VPC to adopt the 2° fate^{3,18,19}.

Reducing *sel-12* activity reduces *lin-12* activity in lateral signalling that specifies the 2° fate of VPCs. First, *sel-12* reduces the effect of activated LIN-12 in the VPCs: all VPCs adopt the

TABLE 2 *sel-12(ar171)* plays a role in the receiving cells

Genotype	Expression of 2° fate/total						VPCs adopting a 2° fate/hermaphrodite (%)
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	
<i>lin-12(n950)</i>	7/7	7/7	7/7	7/7	7/7	7/7	100
<i>lin-12(n950); sel-12(ar171)</i>	0/8	1/8	4/8*	8/8	6/8	2/8†	52
<i>lin-12(n950)</i>	X	11/11	X	X	X	X	100
<i>lin-12(n950); sel-12(ar171)</i>	X	3/10	X	X	X	X	30

Animals were maintained at 20 °C. Early L2 hermaphrodites (as judged by the size of the gonad) were chosen for laser ablation studies. The fates of the VPCs have not been determined at this time; the VPCs become determined many hours later, in the L3 stage¹³. P3.p and P5.p–P8.p were killed with a laser microbeam; the success of this operation was verified 2–3 h later. The following day, the operated animals were mounted for Nomarski microscopy so that the cell lineage of P4.p could be observed directly. In both operated and unoperated animals, vulval fates were scored by directly observing the cell lineage of each VPC. The operated animals were observed until the early L4 stage, to ensure that no divisions were missed.

X indicates cell killed by a laser microbeam. Numbers in each column correspond to the proportion of times a given VPC was observed to adopt the 2° fate (criteria as in ref. 19). All VPCs that did not undergo 2° fates underwent 3° or non-vulval fates, with three exceptions: *, in 1/8 animals examined, P5.p underwent a hybrid (2°/3°) lineage; †, in 2/8 animals examined, P8.p underwent a hybrid (2°/3°) lineage.

2° fate in *lin-12(n950)* hermaphrodites, but only half of the VPCs adopt the 2° fate in *lin-12(n950); sel-12(ar171)* hermaphrodites (Tables 1b and 2). Second, *sel-12* reduces lateral signalling that occurs upon activation of *let-60* Ras. We analysed VPC lineages (data not shown) in *let-60(n1046)* hermaphrodites, in which Ras has been activated by a codon 13 mutation^{20,21}, and in *let-60(n1046); sel-12(ar171)* hermaphrodites. Lateral signalling appears to occur normally in *let-60(n1046)* hermaphrodites, as adjacent VPCs do not adopt the 1° fate (0 of 20 pairs of induced VPCs). In contrast, adjacent VPCs sometimes adopt the 1° fate in *let-60(n1046); sel-12(ar171)* hermaphrodites (4 of 18 pairs), implying that reducing the activity of *sel-12* reduces lateral signalling. Finally, some VPCs adopt the 2° fate in *lin-12(n676n930)* hermaphrodites¹¹. In contrast, VPCs do not adopt the 2° fate in *lin-12(n676n930); sel-12(ar171)* double mutants (data not shown), although we have not tested whether this effect is due to the presence of a second AC.

The genetic interactions of *sel-12* with *lin-12* imply a function for *sel-12* in signalling and/or receiving cells during lateral specification. We have tested whether *sel-12* functions in the receiving end of *lin-12*-mediated cell–cell interactions by performing cell ablation experiments (Table 2). We reasoned that, if all VPCs but one were ablated with a laser microbeam, the fate of the isolated VPC would reflect its intrinsic level of *lin-12* activity in the absence of lateral signal. Thus, in *lin-12(n950)* hermaphrodites, an isolated VPC adopts the 2° fate (Table 2), suggesting that it has a high level of ligand-independent activation of LIN-12 in the VPCs¹⁰. If *sel-12* were to function in one VPC to lower *lin-12* activity in another, then in *lin-12(n950); sel-12(ar171)* hermaphrodites an isolated VPC should also adopt the 2° fate. However, if *sel-12* were to function within a VPC to lower its *lin-12* activity, then in *lin-12(n950); sel-12(ar171)* hermaphrodites an isolated VPC should instead adopt the 3° fate. We observed that in *lin-12(n950); sel-12(ar171)* hermaphrodites, an isolated P4.p often adopts the 3° fate (Table 2), implying that *sel-12* functions within a VPC to lower *lin-12* activity.

We cloned *sel-12* by transformation rescue (Fig. 1 legend), and determined the nucleotide sequence of a full-length cDNA (Genbank accession number U35660). The predicted SEL-12 protein contains multiple potential transmembrane domains (Fig. 1), consistent with its SEL-12 function as a receptor, ligand, channel or membrane structural protein. The SEL-12 protein is evolutionarily conserved. Database searches revealed a high degree of similarity to a sequence of a partial complementary DNA from human brain present on clone T03796, and a low degree of similarity to SPE-4, a protein required for *C. elegans* spermatogenesis²². In addition, SEL-12 is highly similar to S182, which, when mutant, has been implicated in familial early-onset Alzheimer's disease³. The predicted protein sequences of SEL-12, T03796, SPE-4 and S182 are aligned in Fig. 1.

Many different cell fate decisions are specified by *lin-12/Notch* genes in *C. elegans* and *Drosophila*, and in both organisms some of these decisions are critical for neurogenesis. The genetic analysis described here indicates that *sel-12* facilitates *lin-12*-mediated reception of intercellular signals. SEL-12 might be directly involved in *lin-12*-mediated reception, functioning for example as a co-receptor or as a downstream effector that is activated upon LIN-12 activation. Alternatively, *sel-12* may be involved in a more general cellular process such as receptor localization or recycling and hence influence *lin-12* activity indirectly. Although the remarkable conservation of SEL-12 and S182 does not provide any immediate indication of the function of S182 in the Alzheimer's disease process, it is striking that 4 of the 5 mutations found in affected individuals alter amino acids that are identical in SEL-12 and S182 (see Fig. 1). The powerful tools of classical and molecular genetic studies in *C. elegans*, including the ability to identify extragenic suppressors and to generate transgenic lines containing engineered genes, can now be brought to bear on fundamental issues of SEL-12/S182 structure and function. □

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Signalling downstream of activated mammalian Notch

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NOTCH belongs to a family of transmembrane proteins that are widely conserved from flies to vertebrates and are thought to be involved in cell-fate decisions. In *Drosophila*, the *Suppressor of*

hairless (*Su(H)*) gene^{1,2} and genes of the *Enhancer of split* (*E(Spl)*) complex, which encode proteins of the basic helix-loop-helix type^{3,4} have been implicated in the Notch signalling pathway. Mammalian homologues of *E(Spl)*, such as the mouse *Hairy enhancer of split* (*HES-1*)⁵, have been isolated. Both *HES-1* and the intracellular domain of murine Notch (mNotch) are able to block MyoD-induced myogenesis⁵⁻⁷. Here we show that activated forms of mNotch associate with the human analogue of *Su(H)*, KBF2/RBP-J κ (refs 8, 9) and act as transcriptional activators through the KBF2-binding sites of the *HES-1* promoter.

In *Drosophila*, Notch signalling activity is directly responsible for the accumulation of basic helix-loop-helix (bHLH) proteins encoded by the *E(Spl)* complex³. To investigate whether mNotch modulates *HES-1* transcription, we carried out transfection experiments in HeLa cells. mNotch derivatives (Fig. 1e) were cotransfected with a *HES-1-luc* construct (Fig. 1a, d): wild-type (WT) mNotch⁶, three mutants able to suppress induced myogenesis (*IC* (ref. 7), *ICL* (ref. 6) and ΔE (R.K. *et al.*, manuscript submitted)), and inactive mutants (*IC-M2* (ref. 7)

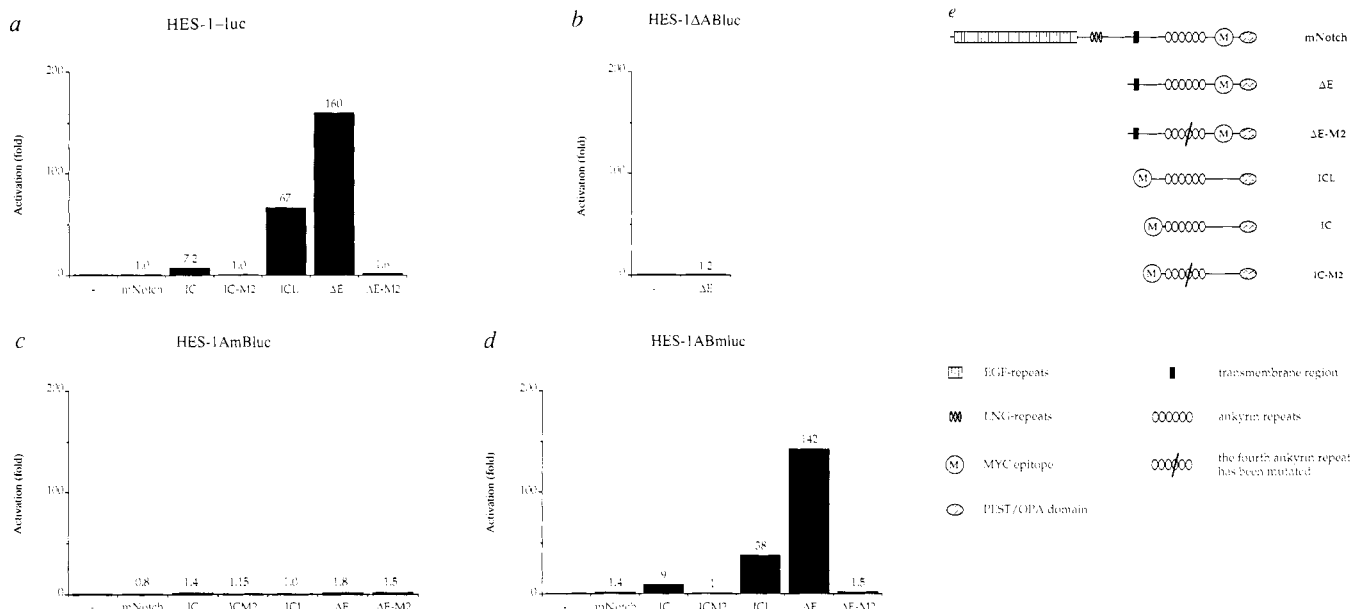
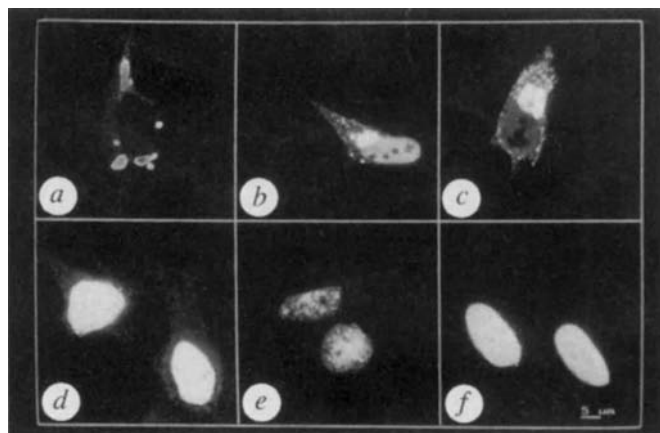


FIG. 1 Transcriptional activation of the *HES-1* promoter by mNotch derivatives. HeLa cells were transfected with a luciferase reporter plasmid alone (symbolized by a dash) or together with various mNotch expression vectors as indicated. **a**, *HES-1-luc* contains the -194 to +160 promoter fragment of the *HES-1* gene cloned upstream of the luciferase gene in the pGL2-basic vector (the A and B KBF2/RBP sites are centred at -81 and -67 respectively from the start site). **b**, *HES-1 Δ ABluc* is derived from *HES-1-luc* by deletion of both A and B RBPJ κ -binding sites. **c**, *HES-1AmBluc* is derived from *HES-1-luc* by mutation of the A site. **d**, *HES-1ABmluc* is derived from *HES-1-luc* by mutation of the B site. The choice of the mutated bases was guided by the results of Tun *et al.*²¹. The luciferase reporter plasmid/mNotch expression vector ratio was (1:2) in all cotransfections. **e**, The mNotch derivatives used: mNotch corresponds to the murine Notch clone described in ref. 6; IC starts at amino acid 1,810 and contains the intracellular domain of mNotch⁷; IC-M2 is a mutant of IC having two amino-acid substitutions in the fourth ankryin repeat that abolish activity in a myogenesis inhibition assay⁷; ICL⁶ is identical to IC except that it contains 57 additional amino acids (1,753 to 1,809) at the N-terminal end of the molecule. ΔE starts at amino acid 1,704, 20 amino acids upstream of the transmembrane region, and goes to the C terminus of the molecule (R.K. *et al.*, submitted); constructs similar to ΔE behave as constitutively activated forms of Notch in *Drosophila* or *Xenopus*²²⁻²⁴; the M2 mutation has been introduced into the ΔE molecule, giving rise to the ΔE -M2. A Myc epitope has been introduced at the N terminus of the IC- and ICL-encoded constructs, and between amino acids 2,193 and 2,194 in the other mNotch constructs^{6,7}.

METHODS. The fragment of the murine *HES-1* gene between -194 and +160 from the start site was isolated by polymerase chain reaction (PCR) using the following oligonucleotides: 5' oligo: 5'-GGGGTACC-CTCAGGCGCGGCCATTGGCC-3' and 3' oligo: 5'-GAAGATCTGCTTACGT-CCTTTTACTTGAC-3'. The amplified fragment was subcloned between the *KpnI* and *BglII* sites of the pGL2 basic vector (Promega) to give *HES-1-luc*. Deletion and mutation of the two KBF2/RBP-J κ -binding sites was accomplished using PCR. The wild-type sequence from -91 to -57 is: 5'-TGAAAGTTACTGTGGGAAAGAAAGTTGGGAAGTTTCACACGAG-3'; the core sequence of the two KBF2/RBP-J κ -binding sites is underlined. The mutagenic oligonucleotides (in the antisense orientation) are: Δ AB, 5'-TCGTGTGAAACTTACAGTAACCTTCA-3'; AmB, 5'-CCAAACTTCTGCAGC-ACAGTAACCTTTC-3'; ABm, 5'-CTCGTGTGAAACTGCAGAACTTCTTTC-3' (substituted bases in bold). The PCR products were cloned between the *KpnI* and *BglII* sites of the pGL2-basic vector, giving rise to the *HES-1 Δ ABluc*, *HES-1AmBluc* and *HES-1ABmluc*. Deletion and mutations were verified by sequencing. HeLa cells were transfected in 6-well plates by the calcium phosphate co-precipitation method, with 400 ng luciferase vector alone or in combination with 800 ng of one of the mNotch expression vectors; 400 ng SV40-*lacZ* construct was included in each transfection as an internal control. Luciferase activity was measured 48 h after transfection in a luminometer (Berthold) and normalized according to the β -galactosidase activity; bars represent the ratio between individual normalized luciferase activities and the normalized activity measured with the luciferase plasmid alone. Data correspond to the average of at least 4 independent experiments. Basal levels of activity were not significantly different between the various luciferase constructs.

FIG. 2 Subcellular localization of the mNotch and RBP3 proteins in transfected HeLa cells. Immunofluorescence analysis of: a, HeLa cells transfected with WT *mNotch* and stained with the 9E10 anti-Myc mAb; b, HeLa cells transfected with ΔE and stained with the 9E10 anti-Myc mAb; c, HeLa cells transfected with ΔE -M2 and stained with the 9E10 anti-Myc mAb; d, HeLa cells transfected with IC and stained with the 9E10 anti-Myc mAb; e, HeLa cells transfected with IC-M2 and stained with the 9E10 anti-Myc mAb; f, HeLa cells transfected with 5'-tagged RBP3 and stained with anti-Flag mAb.

METHODS. HeLa cells were transfected as for Fig. 1 and 46 h after transfection were fixed for 20 min in 3% paraformaldehyde, followed by 10 min in 50 mM NH_4Cl , and permeabilized for 5 min in 0.2% Triton X-100. Cell monolayers were then incubated with an anti-Myc epitope monoclonal antibody (9E10), which detects mNotch, or an anti-Flag antibody (Eastman Kodak Company), which detects RBP3. After washing, antibodies were detected with FITC-conjugated secondary antibody, and nuclei were stained with Hoechst 33258; pictures were taken using a confocal laser scanning microscope (Zeiss).



and ΔE -M2). Whereas wild-type *mNotch* construct shows no stimulatory effect, the IC, ICL and ΔE constructs activate the *HES-1* promoter (Fig. 1a); the IC-M2 and ΔE -M2 mutants were inactive in this assay.

As Su(H) acts downstream of Notch¹ and interacts directly with its intracellular ankyrin repeats¹⁰, we investigated the role of KBF2/RBP-J κ in this transcriptional activation. There are two adjacent KBF2/RBP-J κ -binding sites in the promoter of the *HES-1* gene¹¹, both able to bind the RBP-J κ protein efficiently (Fig. 3 and ref. 12). But in transfection experiments this protein is unable to activate the *HES-1* promoter (not shown). A deletion of these two sites abolishes the response of the promoter to the *mNotch* constructs (Fig. 1b). Furthermore, the two KBF2/RBP-J κ sites are not functionally equivalent because mutation of the B site does not affect activation by IC or ΔE , whereas mutation of the A site abolishes it (Fig. 1c, d).

We examined the subcellular localization of the mNotch and RBP3 (ref. 12) proteins by immunofluorescence of transfected HeLa cells. As in the case of *Drosophila* Notch^{13,14}, most of the mNotch protein is included in cytoplasmic vesicles (Fig. 2a), although some is localized at the plasma membrane. Nuclear staining was observed for ΔE (Fig. 2b) as well as for ΔE -M2 (Fig. 2c), although large amounts of transfected proteins are localized at the plasma membrane and in the cytoplasm. Characterization of the processing of the ΔE mutant, the nuclear translocation of the processed form and its relevance to the biological activity of mNotch will be presented in detail elsewhere (R.K. *et al.*, submitted). The IC (Fig. 2d), IC-M2 (Fig. 2e), ICL (ref. 6, and data not shown) and RBP3 (Fig. 2f) proteins are predominantly nuclear. Thus the transcriptionally active forms of mNotch localize at least partly to the nucleus, where they could act as transcriptional activators.

As *in vitro*-translated products of various *mNotch* derivatives do not bind directly to the KBF2/RBP-J κ binding sites (data not shown), we investigated whether RBP3 could serve as an anchor molecule to allow active forms of mNotch to stimulate transcription of the *HES-1* promoter. KBF2/RBP-J κ has been shown to act as a tether protein in mediating transcriptional activation by the EBNA-2 protein of Epstein-Barr virus (EBV)¹⁵⁻¹⁸. We did gel retardation assays with oligonucleotides containing one intact and one mutated site (ABm and AmB, see Fig. 3 legend for sequences) using nuclear extracts of 293T cells transfected with ΔE , ΔE -M2 or ICL and/or RBP3-expression vectors. RBP3 binds to both oligonucleotides (band labelled RBP3 in Fig. 3a and b). When cells were transfected with myc-tagged ΔE , an additional complex was detectable using the ABm oligonucleotide (complex 1 in Fig. 3a; lanes 7, 8, 10 and 20). Preincubation of these extracts with 9E10 anti-Myc antibody led to the formation of a supershifted complex, appearing in a

RBP3-dose-dependent manner (complex 3 in Fig. 3a; compare lane 12 containing transfected RBP3 with lane 9 containing endogenous KBF2/RBP-J κ), indicating that this complex contains the nuclear form of ΔE . Complex 1 is also supershifted by preincubating the extracts containing Flag-tagged RBP3 with anti-Flag antibody (complex 2 in Fig. 3a, lanes 11 and 22, and data not shown) or with both anti-Myc and anti-Flag antibodies (Fig. 3a: complex 7 in lane 23). We observed similar complexes for wild-type AB oligonucleotide, in which both sites can be occupied by RBP3 (not shown).

These results indicate that KBF2/RBP-J κ (either the endogenous or the transfected protein) can associate with the nuclear form of ΔE to complex with DNA. The activation of the *HES-1* promoter by ΔE in the absence of cotransfected RBP3 can be explained by recruitment of the nuclear form of ΔE by endogenous KBF2/RBP-J κ . Using ICL-transfected cell extracts, it is also possible to detect complexes (Fig. 3a: complex 4 in lanes 16 and 18) supershifted by 9E10 (complex 5 in lane 19) or anti-Flag antibodies (complex 6 in lane 17), but these are weaker than those from ΔE -transfected extracts. ΔE may be naturally processed to give rise to a more physiological and active form of the protein. Extracts from cells transfected with RBP3 and ΔE -M2 constructs give rise to the much weaker complexes 1, 2 and 3 (Fig. 3a, lanes 13-15), suggesting that the lack of transcriptional activity of M2 mutants may be partly due to a lower affinity for KBF2/RBP-J κ . Using the AmB oligonucleotide, weak complexes 1 and 3 (Fig. 3b, lanes 3 and 4) could be detected, suggesting that the affinity for this probe is much lower. These results agree with the transactivation experiments and indicate that the flanking sequences of the KBF2/RBP-J κ -recognition sites are important for binding of the complex between KBF2/RBP-J κ and the nuclear form of ΔE or ICL.

Using an interaction assay¹², we found that labelled *in vitro*-translated ICL protein can be specifically retained on GST-RBP3-coated agarose beads (Fig. 3c, lane 4), confirming that RBP-J κ and an activated form of mNotch can interact even in the absence of DNA.

Our results provide a molecular understanding of how activated forms of Notch might alter gene expression. For example, two oncogenic forms of Notch that result from chromosomal alterations (a translocation in the case of *Tan-1* (ref. 19), and a retroviral insertion in the case of *int-3* (ref. 20)) may give rise to activated forms of Notch like those described here. Another possibility is that after activation of wild-type Notch by ligand binding, processing is followed by nuclear translocation of the intracellular form, which could then be recruited to promoters via the KBF2/RBP-J κ protein to activate transcription of downstream genes such as *HES-1*. Activation of *HES-1*, which encodes a bHLH protein that blocks transcriptional activation

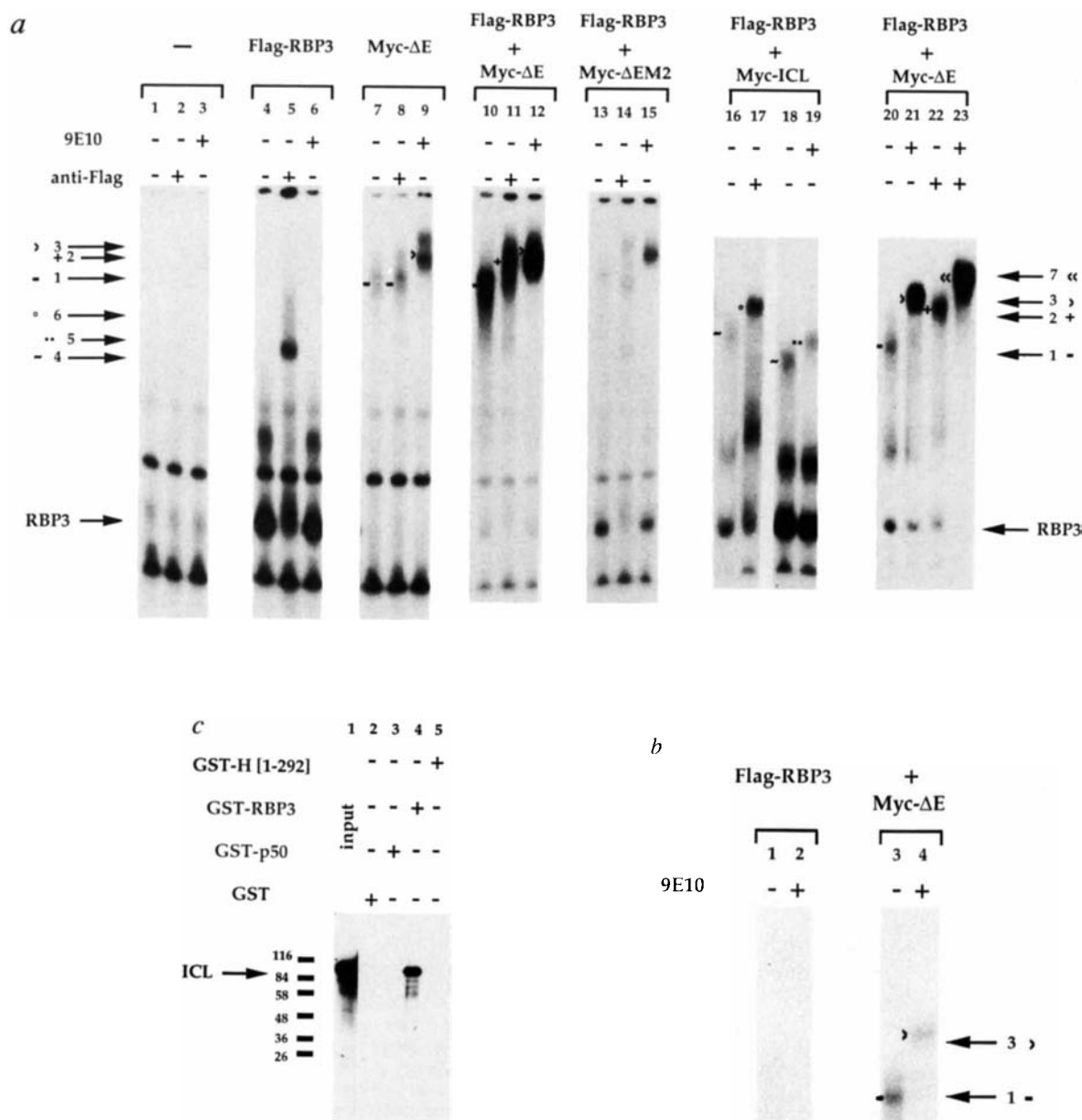


FIG. 3 Electrophoretic mobility shift assay (EMSA) of nuclear extracts derived from transfected 293T cells. **a**, EMSA of nuclear extracts (5 µg) preincubated with anti-Flag antibody (lanes 2, 5, 8, 11, 14, 17, 22), with 9E10 anti-Myc antibody (lanes 3, 6, 9, 12, 15, 19, 21), both antibodies (lane 23) or neither (lanes 1, 4, 7, 10, 13, 16, 20), using the labelled oligonucleotide ABm:

GATCGTTACTGTGGAAAGAAAGTTTCTGTCAGTTTCACAC

CAATGACACCCCTTTCTTTCAAAGACGTCAAAGTGTGCTAG

Note that lanes 1–15 are derived from the same gel, and that lanes 10–15 represent a shorter exposure. Lanes 16–17 and 18–19 are from two different gels. Shifted complexes are numbered 1–7 and are distinguished by symbols. **b**, Using labelled oligonucleotide ABm:

GATCGTTACTGTGCTGCAGAAAGTTTGGGAAGTTTCACAC

CAATGACACGACGTCTTTCAAACCCCTTCAAAGTGTGCTAG

Similar experiments were done with 9E10 anti-Myc antibody or no antibody as indicated. Extracts were derived from cells transfected with expression vectors encoding the indicated proteins, tagged as described in Fig. 2 legend. RBP3 indicates a DNA complex with RBP3 protein. The shifted complexes (numbered 1 to 7, with distinguishing symbols) are described in the text. **c**, *In vitro* interaction between ICL and RBP3 proteins. ³⁵S-methionine-labelled *in vitro*-translated ICL was incubated with glutathione-agarose beads carrying various glutathione S-transferase (GST) fusion proteins as described in ref. 12, except that the wash

buffer contained 400 mM KCl. Control include GST alone (lane 2), GST-NF-κB p50 (a protein known to associate with ankyrin repeats-containing proteins; lane 3), or GST-H[1-292] (lane 5; described in ref. 12). Molecular weight markers are indicated at the left of the gel. The input lane shows the *in vitro*-translated product before incubation with the beads.

METHODS. 293T cells were transfected as described for Fig. 1 with expression vectors encoding RBP3 alone or RBP3 together with mNotch ΔE, ΔE-M2 or ICL, as indicated. Nuclear extracts were prepared as described¹². Equal amounts of proteins were incubated for 1 h at 4 °C in the presence or absence of either a monoclonal antibody (9E10) raised against the Myc epitope or the anti-Flag M2 antibody (Eastman Kodak Company), or both, and then analysed by EMSA using 4% polyacrylamide gels.

by the MyoD protein⁵, could explain the effect of mNotch on myogenesis. □

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Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway

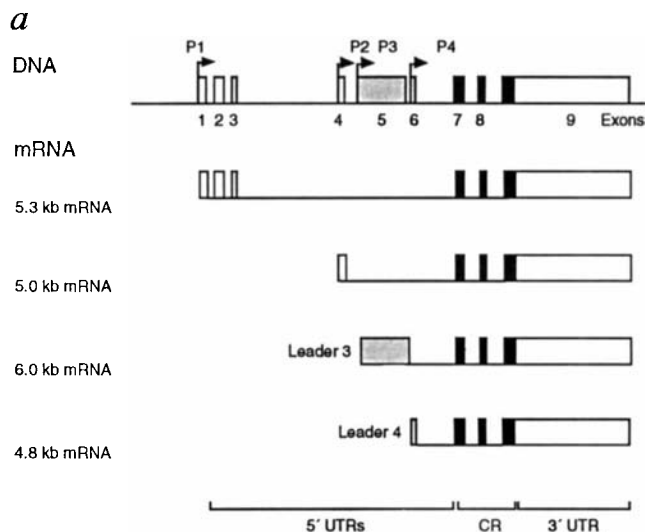
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INSULIN-LIKE growth factor (IGF)-II is important for fetal growth and development¹. The human IGF-II gene generates multiple mature transcripts with different 5' untranslated regions (5'UTRs) but identical coding regions and 3'UTRs². We have previously shown that a minor 4.8-kilobase messenger RNA was engaged in the synthesis of preproIGF-II, and a major 6.0-kb mRNA was untranslated and stored in a 100S ribonucleoprotein particle³. Here we demonstrate that the 6.0-kb mRNA is selectively mobilized and translated in dispersed exponentially growing cells. Translational activation is prevented by rapamycin and mimicked by anisomycin, which suggests that translation of the 6.0-kb mRNA is regulated by the p70^{S6k}/85^{S6k} kinase signalling pathway. Therefore, the minor 4.8-kb mRNA generates a constitutive production of prepro-IGF-II, whereas the major 6.0-kb mRNA provides a post-transcriptionally regulated species.

To establish whether the translational repression of the apparently non-functional 6.0-kb mRNA was controlled by cellular factors or was a consequence of the higher-order RNA structure, we examined the translational status of chimaeric mRNAs containing the 1.2-kb leader 3 *in vitro* and *in vivo* (Fig. 1). Messenger RNAs containing the entire IGF-II leader 3 or 139 nucleotides of the Cap-proximal part of the leader (pΔ3CAT)



b

<i>In vitro</i> (% of pCAT activity)		<i>In vivo</i> (% of pBLCAT activity)	
pCAT	100	pBLCAT	100
pLeader4CAT	43.0 (6.7)	pBLeader4CAT	117 (33)
pLeader3CAT	<0.1 (0.01)	pBLeader3CAT	47 (9)
pΔ3CAT	<0.1 (0.01)	pBLΔ3CAT	78 (32)

FIG. 1 a, Structure and composition of the human IGF-II gene and mRNAs. Exons are represented by boxes and numbered according to ref. 24. Filled boxes mark the coding region for the IGF-II prepropeptide, and sites of transcription initiation are indicated (P1, P2, P3 and P4). Promoter P1 is only active in the adult liver, whereas promoters P2, P3 and P4 are active in the fetus and transformed cells. The composition of the multiple mRNAs is shown below. The 5'UTRs of the predominant 6.0- and 4.8-kb mRNAs, expressed in RD rhabdomyosarcoma cells, are shown in grey. b, Translatability of chimaeric IGF-II leader 3- and leader 4-CAT transcripts *in vitro* and *in vivo*. The results are shown as the mean with standard deviation of three experiments and expressed as a percentage of the pCAT and pBLCAT activities, respectively.

METHODS. Two series of plasmid constructions, pCAT and pBLCAT, were used to generate RNAs for *in vitro* translation in rabbit reticulocyte lysates and for transfection experiments, respectively. pCAT was generated from pUT719, which is a pUC19 derivative containing the T7 RNA polymerase promoter. The pBLCAT construct is identical to pBLCAT2 (ref. 25) and exploits the herpes simplex virus (HSV) thymidine kinase promoter. The IGF-II leader-exons were inserted into the unique BglII and/or XhoI sites upstream of the CAT 5' UTR. RNAs from the pCAT series were prepared as described⁵, but reactions included 0.7 mM m7GpppG (Pharmacia) and 0.35 mM rNTPs to generate m7GpppG-capped transcripts. The amount of full-length RNA was estimated by a comparison of the *in vitro* synthesized RNA with a dilution curve of transfer RNAs and ribosomal RNAs after polyacrylamide gel electrophoresis (PAGE) and staining with toluidine blue. *In vitro* translation of the pCAT-derived RNAs was performed in rabbit reticulocyte lysates according to the manufacturers instructions (MDL Promega and Amersham). The ³⁵S-labelled CAT protein was quantified by instant image analysis (Packard, USA). The decay of the individual transcripts during *in vitro* translation was examined by incubating [α -³²P]-labelled RNA with the lysate for 0, 10, 30 and 60 min at 30 °C before the RNA was isolated and electrophoresed in a denaturing 5% polyacrylamide gel. Transient transfections of exponentially growing human SK-N-MC neuroepithelioma cells were performed as described²⁶. The cells were cotransfected with 20 µg DNA from the pBLCAT series of constructs and 5 µg pCH110 DNA (Pharmacia) containing the *lacZ* gene. CAT and β -galactosidase activities were analysed as described^{27,28}. Moreover, the level of mRNA from each of the constructs was estimated by competitive reverse transcription-polymerase chain reactions (RT-PCR). The competitor was generated by destroying the EcoRI site in the CAT coding region, so that competitor and target could be separated by digesting with EcoRI after PCR amplification.

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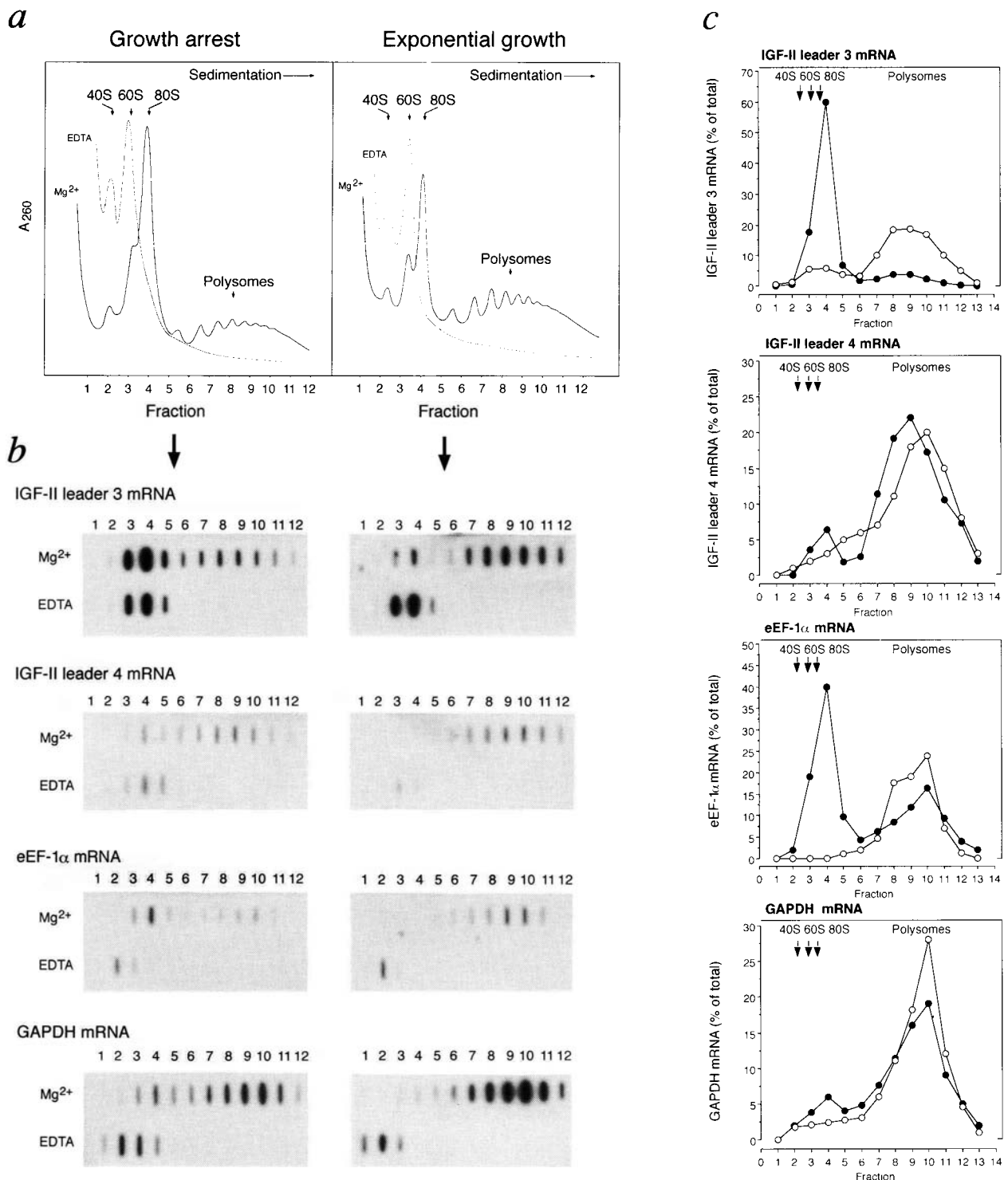


FIG. 2 Growth-dependent translation initiation of IGF-II mRNAs. **a**, A₂₆₀ sedimentation profile of detergent-solubilized cytoplasmic lysates in a 20–47% (w/w) sucrose gradient in 5 mM MgCl₂ (Mg²⁺) or 10 mM EDTA (EDTA) from confluent growth-arrested RD cells (left) and dispersed exponentially growing RD cells (right). **b**, Slot-blot analysis of 6.0 kb (leader 3 mRNA) and 4.8 kb (leader 4 mRNA) IGF-II mRNAs and transcripts encoding eEF-1α and GAPDH in the gradient. **c**, The quantitative analysis of the hybridization signals from the slot-blot analysis. Filled symbols depict signals from growth-arrested cells, and open symbols are the signals from dispersed exponentially growing cells.

METHODS. Growth-arrested (5×10^5 cells per cm²) or exponentially growing (1×10^5 cells per cm²) RD cells were collected and washed in ice-cold phosphate buffered saline with $10 \mu\text{g ml}^{-1}$ cycloheximide before the cells were pelleted. The cell pellet was resuspended in 500 μl lysis buffer (20 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 140 mM KCl, 0.5 mM dithiothreitol, 0.1 mM cycloheximide, 0.5% Nonidet P-40, 1000 units per ml RNasin) and centrifuged at 10,000 g for 10 min. The supernatant was applied to a linear 20–47% sucrose gradient in 20 mM Tris-HCl, pH 8.0, 140 mM KCl with 5 mM

MgCl₂ or 10 mM EDTA, respectively, and centrifuged for 2 h 15 min at 40,000 r.p.m. in a Beckman SW41 rotor. Fractions of ~ 1 ml were collected with concomitant measurement of the absorbance at 260 nm followed by phenol/chloroform extraction. The RNA was applied to a Hybond-N membrane (Amersham) in 10 X SSPE, 6.25 M formaldehyde after heating at 56 °C for 15 min. The filters were hybridized with cDNA probes for IGF-II leaders 3 and 4, eEF-1α mRNA, or GAPDH mRNA. The 265-bp leader 3 cDNA extends from position 891 to position 1155 in exon 5 (ref. 2), and the 96-bp leader 4 cDNA encompasses the complete exon 6 (ref. 2). Eukaryotic EF-1α and GAPDH probes were complementary to the coding regions. The specificity of all cDNA probes was established by northern analysis before their use in slot-blot analysis. Hybridization was performed at 42 °C in 50% formamide, 6 X SSPE, 5 X Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS), $150 \mu\text{g ml}^{-1}$ denatured salmon sperm DNA. Final washes were performed at 65 °C in 0.1 X SSPE with 0.1% SDS. Autoradiography was from 16–72 h, and the hybridization signals were quantified with a BAS2000 bio-imaging analyser (Fuji).

were not translated *in vitro*. In contrast, pBLeader3CAT and pBLΔ3CAT mRNAs were translated in transiently transfected human SK-N-MC neuroepithelioma cells. This analysis suggests that transcripts containing leader 3 are translatable but require the presence of activating cellular factors. During transfections the cells are plated at low densities to ensure logarithmic growth. This led us to examine the translational status of the endogenous IGF-II mRNAs as a function of the cellular growth stage (Fig. 2). Endogenous elongation factor(eEF)-1 α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were used as representatives of the translational apparatus and of glycolysis, respectively. IGF-II-producing rhabdomyosarcoma (RD) cells proliferate in an exponential manner with a doubling time of 20 hours, and growth arrest becomes apparent when the culture reaches confluency at 5×10^5 cells per cm^2 . In growth-arrested cells, 87% of 6.0-kb IGF-II mRNA sedimented as a 100S ribonucleoprotein (RNP) particle and only 13% of the transcripts associated with polysomes. In dispersed exponentially growing cells at a density of 1×10^5 cells per cm^2 the distribution was reversed, and 83% of the transcripts sedimented with the polysomal fractions. The 4.8-kb IGF-II mRNA exhibited a small increase in translation initiation, whereas initiation of GAPDH mRNA was similar in growth-arrested and exponentially grow-

ing cells. As described previously⁴, eEF-1 α redistributed partially from polysomal fractions to mono- and disomes in quiescent cells. The total amount of the transcripts was also dependent on cell density and growth status. Whereas the level of 6.0-kb IGF-II mRNA was 2.6 times higher in growth-arrested cells than in exponentially growing cells, the level of 4.8-kb IGF-II mRNA remained constant. Eukaryotic EF-1 α and GAPDH mRNAs were downregulated 2.0- and 1.4-fold in quiescent cells, respectively. The accumulation of 6.0-kb IGF-II mRNA is a likely consequence of an increased half-life of the mRNA in the 100S particle: the amount of the major 1.8-kb turnover product that appears during endonucleolysis of the 6.0-kb mRNA⁵ was approximately halved in growth-arrested cultures (data not shown).

Phosphorylation of ribosomal protein S6 is increased in proliferating cells, which might facilitate translation of mRNAs with pyrimidine-rich leaders such as those encoding ribosomal proteins, eEF-1 α and eEF-2 (refs 6, 7). In growth-arrested RD cells, phosphorylation of S6 was decreased by a factor of 5 compared with exponentially growing cells, which led us to examine the role of the p70^{S6k}/85^{S6k} pathway in the mobilization of the 6.0-kb IGF-II mRNA. RD cells in logarithmic growth were incubated with rapamycin (20 ng ml^{-1}) to inhibit the signalling pathway

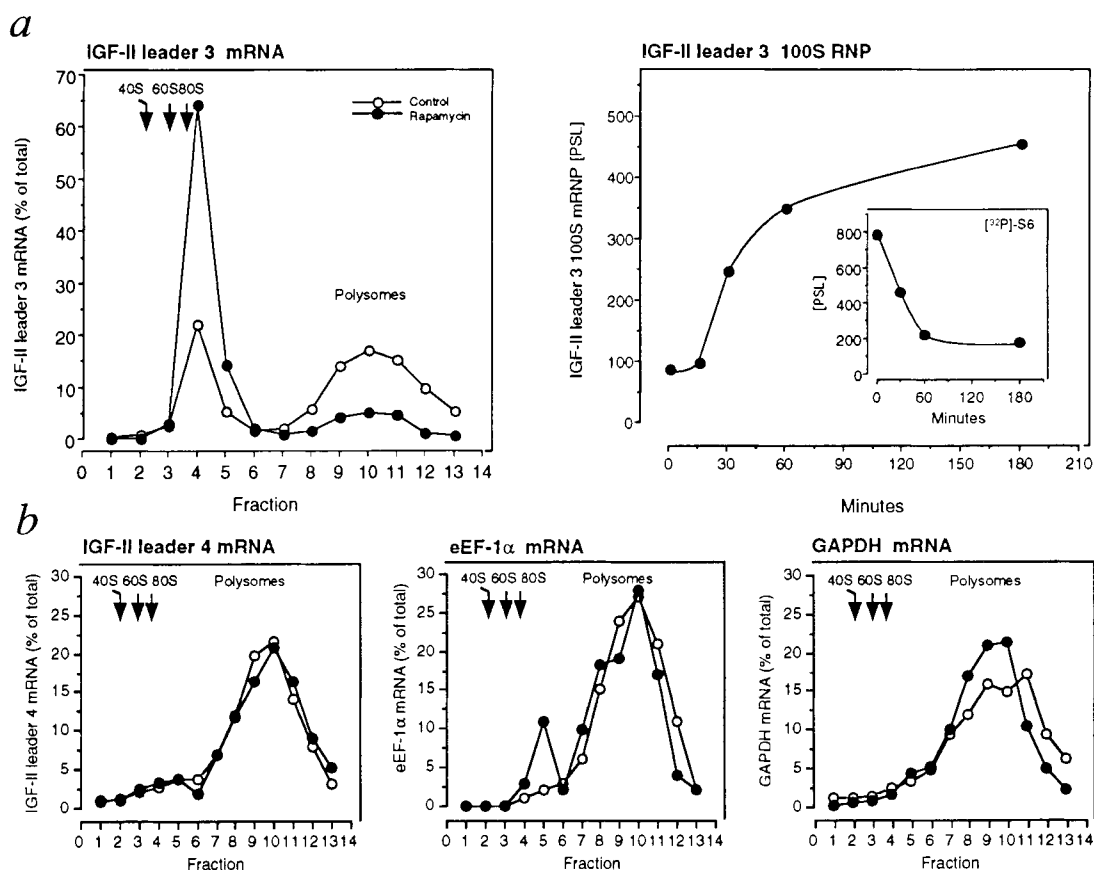


FIG. 3 Rapamycin inhibits translation initiation of 6.0-kb IGF-II mRNA. **a**, Left, the subcellular distribution of 6.0-kb IGF-II mRNA (leader 3 mRNA) in exponentially growing RD-cells incubated for 3 h with (filled circles) or without (open circles) 20 ng ml^{-1} rapamycin. Right, the time dependence of the accumulation of 6.0-kb IGF-II mRNA in 100S RNP following addition of rapamycin, and the insert depicts the level of phosphorylation of ribosomal protein S6. **b**, The distribution of 4.8-kb IGF-II mRNA, eEF-1 α mRNA, and GAPDH mRNA, respectively, after incubation with (filled circles) or without (open circles) 20 ng ml^{-1} rapamycin for 3 h.

METHODS. Exponentially growing RD cells were incubated with 20 ng ml^{-1} rapamycin and collected at the indicated times. The postmitochondrial supernatants were analysed on a linear 20–47% sucrose

gradient and fractions of about 1 ml were collected with concomitant measurement of the absorbance at 260 nm as described in the legend to Fig. 2. When examining the time dependence of the accumulation of transcripts in 100S RNP, fractions 4 and 5 were collected and analysed. Hybridization was performed as described above. Phosphorylation of ribosomal protein S6 was determined essentially as described²⁹. Briefly, RD cells were labelled with $200 \mu\text{Ci ml}^{-1}$ of ^{32}P -orthophosphate for 3 h and incubated with rapamycin for the indicated periods. After collection the cells were lysed, nuclei pelleted, and ribosomes were isolated by centrifugation over a two-layer sucrose cushion²⁹. Ribosomal proteins were analysed by SDS-PAGE and the level of phosphorylated S6 was quantified with a BAS2000 bio-imaging analyser (Fuji), and expressed as photon-stimulated luminescence (PSL).

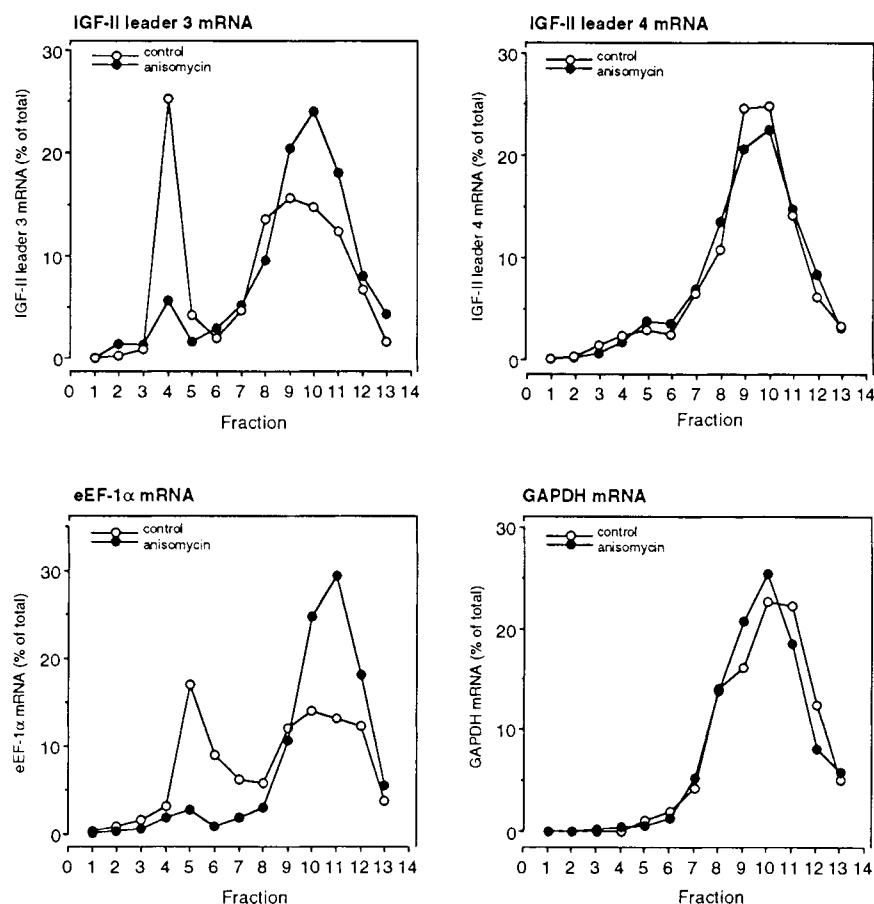


FIG. 4 Anisomycin stimulates translation initiation of 6.0-kb IGF-II mRNA. RD cells at a density of 3×10^5 cells per cm^2 were incubated with (filled circles) or without (open circles) 300 ng ml^{-1} anisomycin for 3 h, and the subcellular distribution of 6.0-kb IGF-II mRNA, 4.8-kb IGF-II mRNA, eEF-1 α mRNA, and GAPDH mRNA was determined as described in the legend to Fig. 2.

that activates the S6 kinase⁸⁻¹⁰ (Fig. 3a). Accumulation of the 6.0-kb IGF-II mRNA in the 100S RNP particle was apparent 30 min after the addition of rapamycin, and correlated with the disappearance of phosphorylated ribosomal protein S6. The effect was virtually complete after 2 hours and was antagonized by a 500-fold molar excess of FK506 (data not shown). Rapamycin had no effect on the translation of either the 4.8-kb IGF-II mRNA or the GAPDH mRNA: both remained associated with polysomes. As described previously⁶, approximately 20% of the eEF-1 α transcripts shifted to mono- and disomes (Fig. 3b). Pactamycin (100 ng ml^{-1}) caused all the examined mRNAs to accumulate as RNPs in exponentially growing RD cells (data not shown), indicating that the accumulation of 6.0-kb IGF-II mRNA is due to inhibition of translation initiation. Anisomycin is an inhibitor of peptidyl transfer, but at subinhibitory concentrations it is an agonist in the S6 kinase pathway^{11,12}. Therefore, we examined the effect of anisomycin (300 ng ml^{-1}) on the translation of the 6.0-kb IGF-II mRNA in RD cells (Fig. 4). Anisomycin caused a redistribution of the 6.0-kb IGF-II mRNA from 100S RNP to polysomes. Eukaryotic EF-1 α mRNA shifted from mono- and disomes to polysomes, whereas the distributions of the 4.8-kb IGF-II mRNA and GAPDH mRNAs were unchanged. Moreover, mobilization of the 6.0-kb IGF-II mRNA by anisomycin was antagonized by pretreatment with rapamycin (data not shown).

Finally, we examined the regulation of transiently expressed chimaeric pBLeader3CAT and pBLeader4CAT mRNAs by comparing CAT activity in rapidly proliferating SK-N-MC cells cultured in fetal calf serum with the activity in slowly propagating cells treated with newborn calf serum. Whereas translation of pBLeader4CAT mRNA was unaffected by stimulation with fetal calf serum, translation of the pBLeader3CAT transcript was increased from 20% (s.e.m. 4%) to 62% (s.e.m. 7%) of the

CAT control in cells stimulated with fetal calf serum (data normalized to transfection efficiency and cell number). We conclude that the two leaders dictate a differential regulation of the IGF-II mRNAs.

Our results show that translation of the major 6.0-kb IGF-II mRNA is correlated with cellular proliferation and regulated by the S6 kinase signalling pathway, whereas the minor 4.8-kb IGF-II mRNA is constitutively translated. Regulation of translation by p70^{S6k}/85^{S6k} has so far only been described for mRNAs encoding components of the translational apparatus. A growth factor mRNA governed by this type of regulation adds a new perspective to the role of p70^{S6k}/85^{S6k}, and presumably phosphorylated ribosomal protein S6, in cellular growth and to the growth-inhibiting effects of rapamycin. Translation of the 6.0-kb IGF-II mRNA appears to be controlled entirely by the S6 kinase pathway, whereas the inhibitory effect of rapamycin on translation of eEF-1 α mRNA is incomplete⁶. The 1.2-kb leader has a high structural potential owing to 24% guanines and 48% cytidines, exhibiting few similarities to the leaders of eEF-1 α and ribosomal protein mRNAs. However, a structural analysis of the 139 nucleotides of the cap-proximal region that promoted translation *in vivo* revealed a displayed pyrimidine island of six nucleotides (data not shown), resembling pyrimidine-rich regions in the leaders encoding eEF-1 α and ribosomal proteins¹³. Additional cytidine-rich regions occur throughout the leader, and the fragment encompassing positions 729-890 has been reported to facilitate translation of a CAT reporter *in vitro*¹⁴. Stretches of pyrimidines are probably not sufficient for responsiveness to p70^{S6k}/85^{S6k} because the constitutively translated 4.8-kb IGF-II mRNA, which exhibits 33 consecutive pyrimidines with multiple CCU repeats, is insensitive to rapamycin treatment. The S6 kinase pathway is distinct from the Ras and MAP kinase pathways^{15,17}, and is activated by stimulation of several

growth factor receptor tyrosine kinases, including the insulin and IGF-I receptors^{18,19}. Because the biological effects of IGF-II are predominantly mediated by the IGF-I receptor¹, IGF-II may promote its own translation by initiating a positive feedback loop. In the fetus, the 6.0-kb IGF-II mRNA appears to be translated during the first trimester of pregnancy in which large amounts of IGF-II are required for placental growth and expansion of organs²⁰. Later in pregnancy, when organs have reached a mature state, translational repression may downregulate IGF-II production²¹. Finally, IGF-II is expressed at high levels in several paediatric tumours and has been demonstrated to promote growth by an autocrine mechanism^{1,22}. Our data suggest how abrogation of the p70^{S6k}/85^{S6k} signalling pathway by rapamycin could be beneficial in the treatment of IGF-II-producing rhabdomyosarcomas²³ and other cancers. □

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Activation of a cell-cycle-regulated histone gene by the oncogenic transcription factor IRF-2

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THE human histone H4 gene *FOI08* is regulated during the cell cycle with a peak in transcription during early S phase^{1,2}. The cell-cycle element (CCE) required for H4 histone activation is a sequence of 11 base pairs that binds a protein factor in electrophoretic mobility shift assays that has been designated histone nuclear factor M (HiNF-M)^{2,3}. Here we report the purification of HiNF-M, and show it to be a protein of relative molecular mass (*M*_r) 48K that is identical to interferon (IFN) regulatory factor 2 (IRF-2), a negative transcriptional regulator of the IFN response⁴. Recombinant IRF-2 (as well as the related protein IRF-1 (ref. 5)) binds the CCE specifically and activates transcription of this H4 histone gene. IRF-2 has been shown to have oncogenic potential⁶, and our results demonstrate a link between IRF-2 and a gene that is functionally coupled to DNA replication and cell-cycle progression at the G1/S phase transition.

To confirm the functional role of HiNF-M, we have introduced a series of specific mutations within the H4 promoter site II, a protein–DNA interaction site established by *in vivo* genomic footprinting⁷. Several protein factors interact with site II (ref. 3), including HiNF-M, HiNF-P and HiNF-D, a multi-component factor containing cyclin A, Cdc2 and an Rb-related protein⁸. Alterations in the HiNF-M binding site decrease H4 gene transcription *in vivo*, whereas mutations in the HiNF-P and HiNF-D sites have no apparent effect (Table 1). These results establish that the HiNF-M binding site, the CCE, is rate limiting for H4 histone gene transcription.

After isolation of HiNF-M from HeLa nuclear extract, several protein bands were visible by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1a). Southwestern blot analysis shows that only a protein of *M*_r 48K binds the CCE specifically and therefore is HiNF-M (Fig. 1b). Four peptides of this protein were microsequenced and the amino acids exactly matched sequences in the human protein IRF-2 (ref. 9).

IRF-2 and IRF-1, negative and positive regulators, respectively, of IFN-β transcription^{4,5}, have homologous DNA-binding domains and show equal affinity for a consensus IRF element¹⁰. They share some homology to ISGF3γ¹¹ and ICSBP¹², and they all bind a sequence called the IFN-stimulated response element (ISRE)^{4,5,11,13} that is found in most IFN-inducible genes. The CCE has high homology to the IRF and ISRE consensus elements, and HiNF-M is efficiently competed for by wild-type ISRE but not mutant ISRE in an electrophoretic mobility shift assay (EMSA) (Fig. 2a). *In vitro* transcribed and translated human IRF-1 and IRF-2 bind the CCE and have the same pattern of oligomer competition as native HiNF-M (Fig. 2a). An additional study to establish that HiNF-M is indeed IRF-2 was performed with IRF antibodies. These antibodies specifically recognize *in vitro* translated IRF-1 and IRF-2 in EMSA by causing a 'super-shift' or 'block-shift', respectively (Fig. 2b). The IRF-2 antibody blocked the formation of native HiNF-M complex from HeLa nuclear extract, but the IRF-1 antibody had no effect on HiNF-M (Fig. 2b).

To ascertain the functional roles of IRF-1 and IRF-2, we performed transient transfection experiments comparing wild-type and HiNF-M mutant H4 promoter constructs in different

TABLE 1 Relative CAT activity of HiNF mutants

Construct	Sequence	Factor binding			Relative CAT activity (%)
		M	P	D	
Wild type	CGCTTTTCGGTTTTCATCTGGTCCGATAC	+	+	+	100.0
M-mutant	-----CA-----	-	+	+	35.0 ± 2.3
P-mutant	-----GTCGAATGC--	+	-	+	128.4 ± 51.1
D-mutant	-----C--G-TC-----CC--	+	+	-	90.9 ± 21.5

The cell-cycle element is rate limiting for H4 gene transcription. Transient transfections were performed in 100-mm dishes of HeLa cells with 20 µg of wild-type² or mutant (F.A., manuscript in preparation) H4 promoter CAT constructs by the calcium phosphate coprecipitation method²². The sequence of the H4 promoter regions for each construct are shown (a dash indicates identical sequence) and the cell-cycle element is underlined. The binding of HiNF-M, P and D to the various fragments in an EMSA is indicated. The percentage of maximal CAT activity²² of each reporter plasmid is shown together with the standard deviation.

cell types. In hamster ts13 and monkey COS7 cells, transcription from the mutant construct is two- to threefold lower than the wildtype (Fig. 3a), similar to the results presented in Table 1. However, in *IRF-1*^{-/-} *IRF-2*^{-/-} (double knockout (DKO)) or *IRF-2*^{-/-} cells, there is no change in transcription from the wild-type or mutant constructs. These results strongly suggest that the IRFs are functionally active on the histone H4 promoter. Furthermore, exogenous IRF-1 and IRF-2 stimulate transcription from wild-type H4 promoters in all cell types tested, but no stimulation is seen with reporter constructs containing specific CCE mutations (Fig. 3b).

Titration of the IRF-2 plasmid shows that we have obtained a maximal level of H4 promoter stimulation (Fig. 3c). A control experiment with an IFN-β promoter-chloramphenicol acetyl transferase (CAT) construct confirms the previously reported result¹⁴: IRF-1 stimulates IFN-β transcription; IRF-2 alone has no effect on IFN-β expression; and coexpression of IRF-1 and IRF-2 shows an inhibition of IRF-1-mediated activation (Fig. 3d). Coexpression of IRF-1 and IRF-2 with the H4 promoter showed no change from expression of IRF-1 or IRF-2 alone (data not shown). Therefore, the effects of IRF-1 and IRF-2 vary with promoter context. This is the first demonstration that

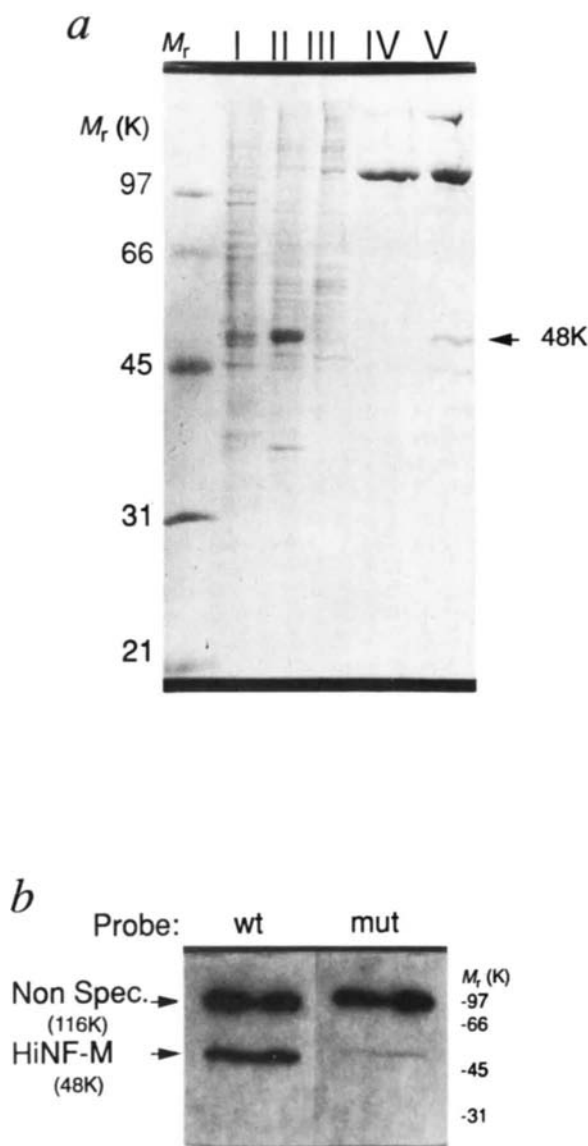


FIG. 1 Isolation of HiNF-M. a, 10% SDS-PAGE²³ of 4 µg each of HiNF-M chromatography fractions followed by Coomassie blue staining. *M_r* standards are shown on the left; lane I, HeLa nuclear extract; lane II, phosphocellulose P-11 pool; lane III, heparin-agarose pool; lane IV, DNA-affinity column I pool; and V, DNA-affinity column II pool. b, Southwestern blot of affinity purified HiNF-M probed with wild-type (wt) or mutant (mut) concatamerized CCE oligomer.

METHODS. a, Nuclear extracts from exponentially growing HeLa S3 cells were prepared as described previously³. Phosphocellulose and heparin agarose chromatography was performed as described previously³ except that HiNF-M was eluted with a gradient of buffer A100 to A1000 (25 mM HEPES, 0.2 mM EDTA, 1 mM DTT, 20% glycerol, 0.01% Nonidet P-40 plus 100 or 1000 mM KCl, respectively). Poly (dGdC) (25 µg ml⁻¹) was added to fraction III before loading on a DNA-affinity column prepared as described²² using concatamerized wild-type CCE oligomer (below). The presence of HiNF-M in column fractions was followed by the binding of factor M to multimerized CCE in a nitrocellulose filter binding assay²⁴ and later confirmed by an EMSA. b, Fraction IV HiNF-M (3 µg) was loaded into parallel SDS-PAGE lanes and electroblotted to polyvinylidene difluoride membrane. The blots were renatured overnight in buffer A100 plus 5% non-fat milk and rinsed briefly in buffer A100 plus 0.25% non-fat milk before overnight incubation with 0.25 × 10⁶ c.p.m. ml⁻¹ ³²P-labelled concatamerized CCE-wt or CCE-mut and 10 µg ml⁻¹ poly(dGdC) in buffer A100. The blots were rinsed in buffer A100 4 times for 5 min before autoradiography. All procedures were performed at room temperature. CCE-wt is GATCCCGCGCGCTTTCAGGTTTCA, and CCE-mut is GATCCCGCGCGCTTTCAGGTTTCA.

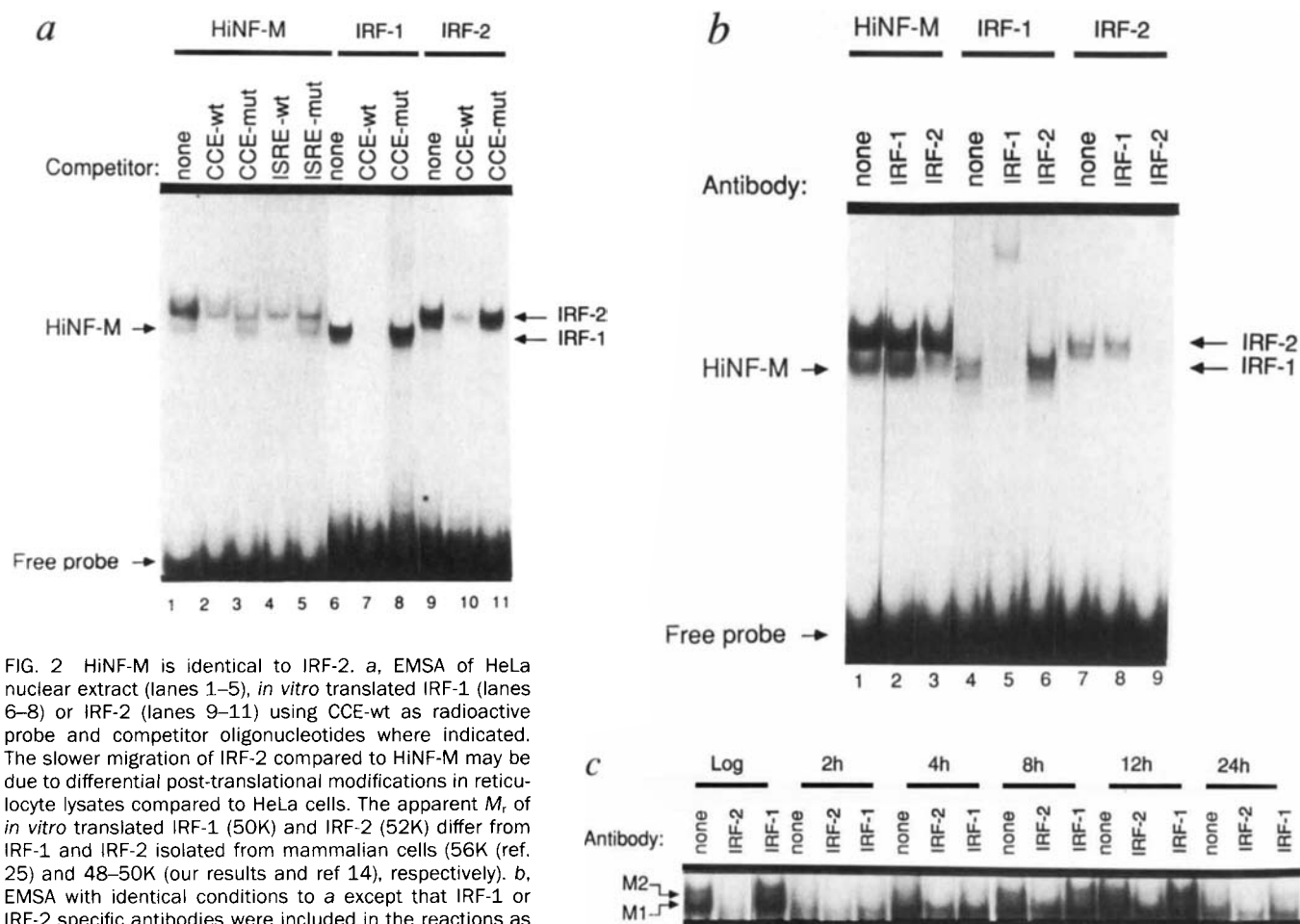


FIG. 2 HiNF-M is identical to IRF-2. *a*, EMSA of HeLa nuclear extract (lanes 1–5), *in vitro* translated IRF-1 (lanes 6–8) or IRF-2 (lanes 9–11) using CCE-wt as radioactive probe and competitor oligonucleotides where indicated. The slower migration of IRF-2 compared to HiNF-M may be due to differential post-translational modifications in reticulocyte lysates compared to HeLa cells. The apparent M_r of *in vitro* translated IRF-1 (50K) and IRF-2 (52K) differ from IRF-1 and IRF-2 isolated from mammalian cells (56K (ref. 25) and 48–50K (our results and ref 14), respectively). *b*, EMSA with identical conditions to *a* except that IRF-1 or IRF-2 specific antibodies were included in the reactions as indicated. *c*, EMSA of nuclear extracts from log-phase or isoleucine-deprived and subsequently cytokine-stimulated FDC-P1 cells¹⁹ (times (h) after cytokine stimulation are indicated) in the presence of IRF-2 or IRF-1 specific antibodies, as indicated. The two forms of HiNF-M (demonstrated by specific oligonucleotide competition behaviour¹⁹) present in log-phase cells are indicated as M1 and M2. METHODS. EMSA was performed as described previously³ with 5 μ g of HeLa or FDC-P1 nuclear extract^{3,19}, 10 fmol ³²P-labelled double-stranded CCE oligomer³, 2 μ g poly(dGdC), and 1 pmol of unlabelled competitor DNA where indicated. The ISRE (AAGTACTTTTCAGTTTCATAT-

TACTCTA) and ISRE mutant (AAGTACTTTTCAGTGGTCTATTACTCTA) oligonucleotides were purchased from Santa Cruz Biotechnology. Where indicated, 1 μ l of *in vitro* transcribed and translated pcDNA1-IRF-1 or pcDNA1-IRF-2 (ref. 14), prepared in a rabbit reticulocyte lysate according to the manufacturer (Promega), and 5 pmol of competitor DNA were used. Antibodies were generated in rabbits against peptides of amino acids 226–242 (IRF-1) and 333–349 (IRF-2) (Research Genetics) and 1.0 μ g of the IgG fractions were added as indicated.

IRF-2 can activate transcription and could provide a physiological role for the known latent activation domain of IRF-2 (ref. 15). IRF-2 now joins the ranks of other transcription factors with dual activator/repressor functions (such as YY1, RAP-1, Dorsal)^{16–18}.

Further evidence for the importance of IRF-2 in H4 gene regulation is shown in Figs 2c and 3a. We have previously shown two distinct HiNF-M complexes in FDC-P1 myeloid stem cells following isoleucine deprivation and subsequent cytokine stimulation¹⁹. These two forms of HiNF-M (designated M1 and M2) are differentially regulated, with M2 levels increasing during the G1/S progression¹⁹ (Fig. 2c). Both M1 and M2 are immunoreactive with IRF-2-specific antibodies but not IRF-1 antibodies (Fig. 2c). M2 may represent a post-translational modification of IRF-2 or the partnering of IRF-2 with another factor during the cell cycle. In addition, *IRF-2*^{-/-} and the DKO cells both have the same level of activity when transfected with the wild-type or mutant H4 constructs (Fig. 3a). This suggests that IRF-1 cannot compensate for the absence of IRF-2 to activate the histone gene in the *IRF-2*^{-/-} cells.

The surprising result that IRFs interact with this histone H4 promoter brings a new dimension to the cell-cycle regulation of

this gene. This interaction may not be unique to the *FOI08* gene, as there are potential IRF binding sites in other histone H4 genes³. IRFs have been shown to be affected by a broad range of physiological mediators, including IFN- α/β , IFN- γ , leukaemia inhibitory factor, interleukin (IL)-1 and IL-6 (refs 5, 20, 21). Combined with the adjacent binding of a cyclin A/Cdc2 complex⁸, it appears that multiple signalling pathways converge on this cell-cycle regulatory element. The results presented here may also provide a basis for the demonstrated oncogenic potential of IRF-2 (ref. 6). □

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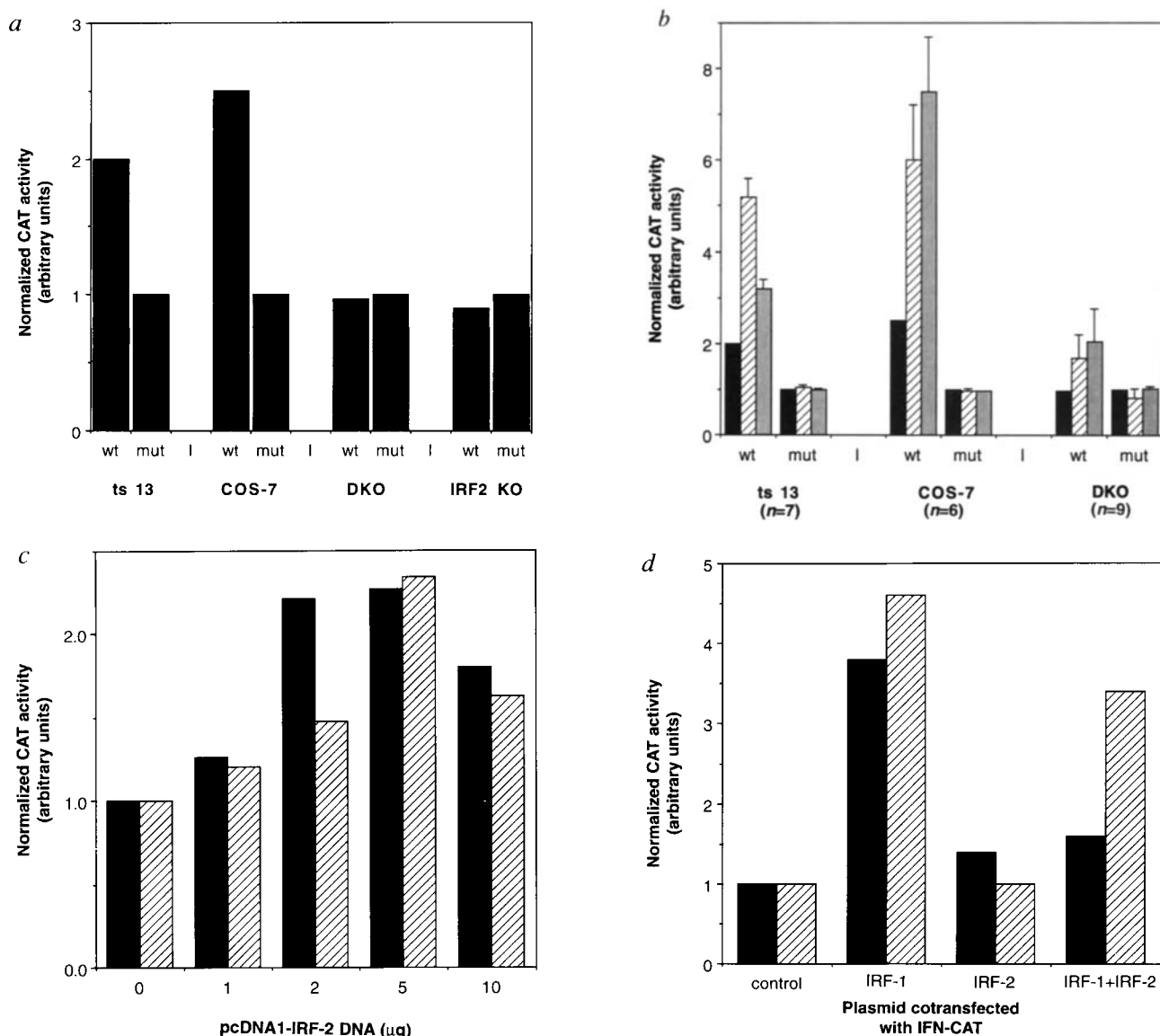


FIG. 3 IRF-2 and IRF-1 stimulate histone H4 transcription. *a*, Transient transfections of wild-type (wt) or M-mutant (mut) H4 promoter-CAT reporter plasmids (Table 1) in hamster ts13, monkey COS7, mouse IRF-1^{-/-} IRF-2^{-/-} (DKO)²⁶, or IRF-2^{-/-} cells²⁷. *b*, Transient cotransfections of wt or mut H4-CAT constructs with control (black bars), IRF-2 (striped bars) or IRF-1 (grey bars) plasmids. The number (*n*) of experiments for each cell type is indicated and the results are shown $\pm$ s.d. *c*, Transient cotransfections of ts13 (black bars) or DKO (striped bars) cells with wild-type H4 promoter-CAT and the indicated amount of pcDNA1-IRF-2. *d*, Transient cotransfections of COS7 (black bars) or DKO (striped bars) cells with the -104 IFN- β promoter-CAT reporter plasmid¹⁴ with control, IRF-1, IRF-2, or both IRF-1 and IRF-2, as indicated. METHODS. Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected either by the calcium

phosphate coprecipitation method (ts13, DKO and IRF-2^{-/-} cells)^{22,28} or the DEAE dextran method (COS7 cells)²⁹. Plates (100 mm) were transfected with 5 μ g reporter plasmid (wild-type or M-mutant H4-CAT (Table 1) or -104 IFN- β CAT¹⁴), 5 μ g expression plasmid (pcDNA1-IRF-2 (ref. 14), pcDNA1-IRF-1 (ref. 14) or 5 μ g of each), 2 μ g pSV- β -gal²⁸ (ts13) or RSV-LUC²² (COS7) and pcDNA1 vector for a total of 17 μ g DNA. CAT levels measured 48 h after transfection were corrected for β -gal or luciferase activity^{22,28}. In some COS7 experiments shown in *b*, pXM-IRF-2 or pXM-IRF-1 expression plasmids¹⁴ along with the pXM vector control were used in place of the pcDNA1 constructs. In *a* and *b*, the CAT activity from the mutant construct in each cell type was given a value of 1.0, and other results were normalized to this. In *c* and *d*, CAT activity from the pcDNA1 vector experiments was given a value of 1.0.

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Getting equipped

Product review your indoor laboratory outfitter, supplies information on automated liquid systems, new benchtop autoclaves and centrifuges, an ultraviolet transilluminator, and a automatic gas-supply switching system.

THE new 1995-96 Thermolyne products catalogue for life science laboratories features the latest laboratory products for heating, temperature control, mixing, stirring, thermal cycling, cryobiological storage, and



Thermolyne life science catalogue.

more (*Reader Service No. 100*). The manufacturer states that it is dedicated to technologically advanced equipment designed and engineered for superior accuracy, performance and dependability.

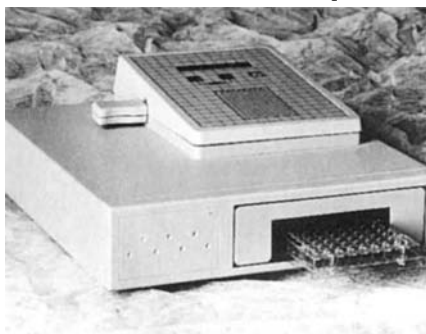
Owl's Gator **horizontal electrophoresis system** offers larger format DNA and RNA separations (*Reader Service No. 101*). Available in three gel sizes, it features leak-proof gel casting, with no tape; gasketed end gates that fit snugly into the UV-transmissible gel tray, forming a tight seal; and a built-in, three-point levelling system to ensure uniform gel thickness. With multiple comb slots, this unit offers maximum versatility to accommodate up to four series of samples on the same gel. Systems are fully-equipped with three combs, UVT gel tray, a safety lid with attached power cords, corrosion- and damage-protected platinum electrodes, an evaporation gap to prevent condensation, no-skid rubber feet and optional buffer exchange ports for extended runs without buffer depletion.

New from Appropriate Technical Resources is the RKVS or RKVSD Rotamix, which provides **end-over-end rotation for all types of mixing and extraction protocols** in the laboratory (*Reader Service No. 102*). Rotamix is designed for quiet operation up

to 80 r.p.m. and is constructed of grey epoxy-coated steel. There are interchangeable 30-cm rods for tubes from 1.5 ml to 50 ml. A large LCD screen provides visual display of the rotation speed.

Automatic action

Optometrics offers Euro Biosystems' PAS, designed to provide a unique method for **microplate washing** (*Reader Service No. 103*). This plug-in module allows the user to optimize wash protocols. To programme the washer, the unit only needs to be plugged into the PAS module. Each module is specific for



The PAS microplate washing system.

each application and can be packaged with reagent kits, reducing procedural errors and increasing reliability. The system will work without the need for special bottles.

Cambio has launched the BioGrid **automated benchtop gridding machine**, the latest product release from BioRobotics (*Reader Service No. 104*). It can produce four 22-cm filters from 24 microtitre plates, of either the 96- or 384-well format, in under 15 min. Set-up time is minimized as the microtitre plates are loaded onto the machine in a plastic rack, which can be stored at low temperature. Plates are automatically drawn out of the rack in pairs, the lids are lifted, and a precision arm transfers samples to the fil-

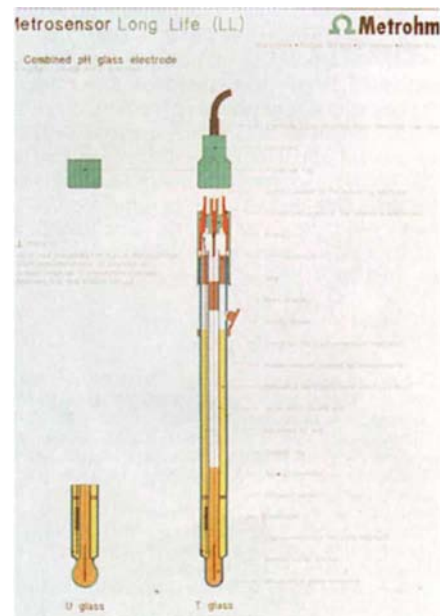


Cambio's benchtop gridding machine.

ters with either a 384- or 96-pin tool. The filter is then moved within the gridding area and the process is repeated from the next microtitre plate. Between plates, the tool is cleaned in a solution of 70 per cent ethanol and dried with hot air, a method which is said to be highly reliable. The instrument measures 55 cm × 56 cm × 50 cm (L × W × H) and therefore fits easily on a laboratory bench. Also available from the company is BioFill, for accurate filling of microtitre plates in the 96- and 384-well format. Plates can be filled in around 20 s.

Electrochemistry

Metrohm has introduced Long Life LL **pH electrodes** with new internal buffers to improve the accuracy of measurements performed with varying temperatures (*Reader Service No. 105*). This is said to be advantageous if calibration is performed at room temperature and measurements have to be conducted at elevated temperatures. The reference electrode is equipped with the reference element in the form of a cartridge so that the reference electrode is no longer saturated with AgCl as with the conventional reference element. This not only improves the measurement accuracy, but also increases the lifetime of the diaphragm. All electrodes are fitted with an alkali-resistant diaphragm. The diaphragm diameter is between 0.8 mm and 1.0 mm, depending on electrode model. The temperature application range of the combined pH electrodes fitted with Ag/AgCl cartridges is 0-80 °C.



Metrohm's Long Life LL pH electrodes.

Whatman offers an eight-page brochure featuring a series of innovative **microprocessor-based pH/mV, conductivity, ORP and temperature instruments** (Reader Service No.



Meters for measuring pH, mV and °C

106). It contains a comprehensive listing of over 20 hand-held and tester-type meters, including complete product descriptions and detailed specifications, software, accessory items, pricing and ordering information.

The new system 100 **cryoware** from Nalgene is designed to save money and available freezer space by increasing storage capacity by 23 per cent over 81-place boxes (Reader Service No. 107). The system consist of two sizes of gasketed vials that can be centrifuged up to 8,000g, a storage box that holds 100 vials, and a 25-place vial holder with increased spacing around the holes for easy vial retrieval. Vials are moulded of polypropylene and use a silicone gasket to ensure leakproof performance in a micro-centrifuge and during shipment or transport. The centrifugation capability of these vials is designed to eliminate the need to transfer the contents to a centrifugation tube after thawing. They have external threading for aseptic technique, a white marking area,



Nalgene's system 100 cryoware.

graduations and a printed fill line. Available in 1.0- and 1.5-ml sizes, the vials come radiation sterilized and certified sterile. The cryobox has a usable temperature range of -196 °C to 121 °C. It has a transparent cover for easy viewing of the contents and a numerical grid for vial identification.

Fluid features

Now available from Karlstein BioLogic are the Pericor VS double-headed **peristaltic pumps**, designed to be pulsation free by utilizing two roller wheels that are positioned parallel to the niche of the other wheel (Reader Service No. 108). A prefabricated set of tubing assures that dimensions on the suction and pressure side are coordinated for precise flow dimensions. The manufacturer states that the pulsation peak is reduced by more than fifty per cent. The elliptical design of the tubing saddle enables the build up and release of the fluid transportation chamber of the tube, which results in an additional reduction of the pulsation peak. Pumps have three rollers in the roller wheel,



KBL's Pericor VS double-headed pump.

which is said to ensure an absolute separation of the suction and the pressure side. Easily exchangeable roller wheels with six or eight rollers are available, and are said to bring the pulse down to zero.

Gilson offers a new four-page, full-colour brochure featuring its model 215 **liquid**

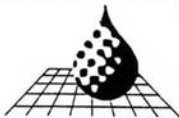
handler, which has been designed for fast, efficient sample transfer, as well as septum-piercing ability for enhanced laboratory safety (Reader Service No. 109). This robotic liquid handler will automate any number of manual liquid handling procedures and sample preparation protocols. It will pierce Vacutainer tube septa or other equivalent thick septa, assuring a high degree of safety by dramatically reducing exposure of laboratory personnel to potentially hazardous or infectious samples (nonpiercing probes are also available). The robot is fully programmable and performs with a positional accuracy of ± 0.5 mm in the x and y axes, with a repeatability of ± 0.25 mm, indicating its ability to access repeatedly very small targets such as microtitre plate wells. The model 215 has an arm speed of about 40 cm s^{-1} and flow rates up to 40 ml min^{-1} , with an integral dilution pump to increase sample throughput. A built-in, liquid-level sensing probe is designed to ensure that sample contamination from carryover is minimized.

Superheated

The Falcon 30, a new **microprocessor controlled portable autoclave**, is now available from LTE Scientific (Reader Service No. 110). It features a rectangular work chamber, which is said to offer considerable advantages over cylindrical models making full use of its 30-l capacity — the stainless steel work chamber is tall enough to accommodate one litre flasks. The unit utilizes a 'keep warm' feature, which allows media to be held in a liquid state until ready for use. The microprocessor control and monitoring system is designed to make the autoclave fully automatic with up to eight load cycles available. Once a cycle is initiated, each stage is clearly indicated on an illuminated mimic diagram. Also included is a built-in, twin-channel printer that records the temperature together with other information, such as batch codes and programme title.

John Godrich has introduced the PrimaClave 250 **benchtop autoclave** (Reader Service No.

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PRODUCT REVIEW

111). It has been designed to cover all requirements of the British Standards as laid down for pressure vessels, autoclaves and health service requirements. There is a 25.4 cm diameter chamber with a 20-l capacity, preset programmes (including a drying cycle), adjustable shelf arrangements for flexibility and there is a printer recorder that can be fitted to document sterilization cycles. The manufacturer states that the 250 is designed for quick turn around and to offer the user easy operation at low budget cost.



John Godrich's PrimaClave 250 benchtop autoclave.

Spin it down

IEC's Centra-CL4, available from Life Science International, is a **benchtop centrifuge** specifically designed to provide high throughput and error-free sample preparation for clinical applications (*Reader Service No. 112*). It has a maximum speed of 6,000 r.p.m. and a capacity for 48 tubes in safety sealed buckets, for aerosol containment of blood and urine specimens. The three-programme memory is designed to give greater user flexibility and programmes can be locked to prevent unauthorized changes to preset parameters. Designed individually to eliminate the errors that arise when estimating the acceleration time, a unique timer enhances accuracy and reproducibility as the timer can be programmed to start once the rotor reaches 95 per cent of set speed. Separate rotors for microplates and different sizes of sample tubes mean that it can also be adapted for use in the tissue culture laboratory.

Heraeus claims its new Biofuge Fresco to be the

smallest **refrigerated benchtop centrifuge** available (*Reader Service No. 113*). It measures 29 × 45 cm and is said to be an ideal low-cost personal centrifuge for microlitre separation tasks in biochemistry and molecular biology. There is a powerful standstill cooling system adjustable between 0 and 40 °C, which maintains the constant sample temperature essential to most biochemical and molecular biological applications. Programmable microprocessor control, through an ergonomically designed keypad, is designed to optimize reproducibility.

Labware

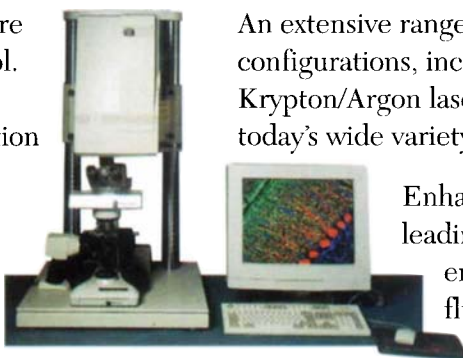
IsoTherm, a two component system from Eppendorf, consists of a **cold pack and tube holder** that can be ordered together in four different versions (*Reader Service No. 114*). There are 0.5 ml and a 1.5- to 2.0-ml tubes, each for 0 °C or -21 °C. The IsoTherm system is designed for 0.5 ml and 1.5- to 2.0-ml regular or safe-lock microcentrifuge tubes and 1.7-ml screw-cap tubes. The IsoRack is an autoclavable tube holder, which resists temperatures down to -200 °C and can be centrifuged up to 2,000g in a microtitre plate rotor. The IsoPack is responsible for cooling, is chemically resistant and contains a nontoxic, biodegradable

If you want power,

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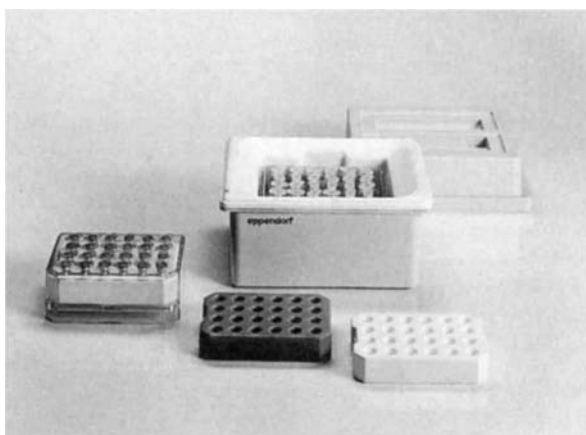
The new Bio-Rad MRC-1024 gives you more power, more sensitivity and far more control. With LaserSharp software, it delivers true simultaneous three-channel (24bit) acquisition and display.

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Eppendorf's tube cooling accessories.

coolant. IsoSafe is designed to keep the other components cool for at least 6 h when precooled to 0 °C for at least 3 h when precooled to -21 °C.

Thermotron has introduced the FZU-21, a 0.6 m³ upright freezer for -86 °C storage of biomedical specimens (*Reader Service No 115*). The company's freezers use HFC refrigerants and refrigeration compressors are sized to provide rapid temperature

pull-downs and quick temperature recovery after door opening. A cleanable, reusable filter protects the machinery from excessive dust build-up which can cause overheating. There is a frost reduction system, and lift-off, inner-compartment doors can be removed for periodic defrosting. The model FZU-21 comes complete with a microprocessor-based control and alarm system to control accurately the temperature and provide audible and visual indication of out of tolerance conditions. The instrument

monitors crucial functions: power, battery back-up, open-sensor protection, door ajar, filter function and high/low temperature limits.

The compact FotoDyne Foto/Phoresis **ultra-violet transilluminator** is designed for safe visualization and photography of DNA minigels (*Reader Service No. 116*). This unit is now capable of doing mutagenesis experiments and other applications. It not only

accommodates minigels, but also a standard 100-mm Petri plate in the UV-light path. It can hold a camera with photographic hood in place while shielding the user from accidental exposure to UV light. An interlocking safety cover makes it possible to view safely the subject without wearing UV-blocking glasses.



Foto/Phoresis.

Labsystem's Cliniplate is designed to be a high-quality, solid, **96-well microplate** (*Reader Service No. 117*). Constructed of clear polystyrene, these plates are said to have excellent optical and binding precision, making them suitable for enzyme linked immunoassays, as well as many other applications. The wells of this plate are raised to reduce the chance of well-to-well contamination. Plates are designed to fit all standard readers and instrumentation, and have a 450-μl capacity. Two binding characteristics are available, 'universal' binding (untreated) and 'enhance' binding (treated). Both binding surfaces are avail-

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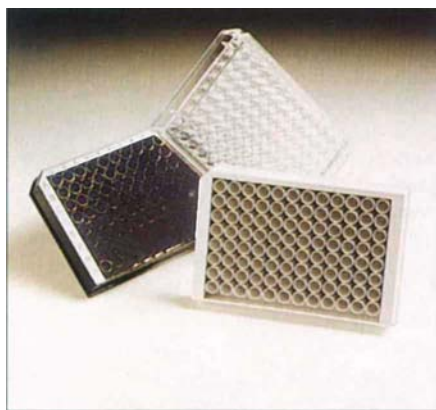
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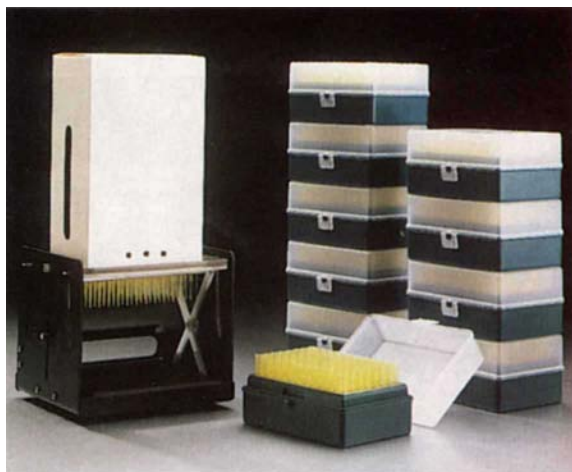
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LabSystems' 96-well microtitre plates.

able with a flat bottom so that protein binding is uniform within the plate and between the plate. Plates are available in black and white formats, as well as streptavidin-coated. The manufacturer states that it uses stringent quality control and manufacturing techniques to ensure high optical quality.

Alpha Laboratories now offers ATL-AS, a system designed for **loading pipette tips into empty racks** within the laboratory (Reader Service No. 118). It is said to be capable of refilling ten empty racks with



Alpha Laboratories' pipette tip loading apparatus.

tips in under sixty seconds, this system provides an economical, recyclable alternative for pipette tip replacement.

Gas products

Matheson Gas Products has introduced the Gen-Switch, a system that maintains continuous flow by **automatically switching from a depleted gas supply to a backup source** (Reader Service No. 119). Engineered to switch from a Matheson nitrogen or zero air gas generators to a standby gas cylinder, the device ensures instrumentation, samples and processes are protected from an interrupted supply of gas. Pressure sensors monitor gas pressure of an in-house line and switch to a reserve cylinder of gas when the pressure drops below a user-specified preset level. An alarm will sound and the status of the front-panel indicator light will change, alerting personnel of the switchover. When the primary gas source is returned to operation, the unit will switch back to the original source at the push of a button. A pressure sensor also monitors the backup supply pressure — delivery pressure range is from 0 to 1,030 kPa.

Packard Instruments has announced the introduction of its new 9000 series **hydrogen generators for gas chromatography** (Reader Service No. 120). The new family contains three models and can produce pure hydrogen at 0 to 500 cc min⁻¹. These generators offer silent, continuous operation at low pressures from 202.6 to 413.6 kPa. Designed to eliminate high-pressure gas cylinders from the laboratory, the 9000 series features micro-processor controlled safety software. Gas is only produced on demand and the systems are exempt from OSHA and NFPA regulations. Solid polymer electrolyte eliminates the use of liquid electrolytes from the hydrogen generators, and the man-

ufacturer states that there is no need to replenish the system with caustic electrolyte to produce ultrapure hydrogen.

Savant's DVG50 **digital vacuum gauge** is used to monitor performance and efficiency of vacuum systems (Reader Service No. 121). It can be used diagnostically to determine whether a vacuum system is free from leaks; verify that a required vacuum level is present (for example, sufficient vacuum exists for a procedure, such as lyophilization); or monitor progress during a vacuum evaporation run. This compact gauge covers a wide range of vacuum levels, from 50 torr down to 1 millitorr. Vacuum level is shown on a large LED display. The device comes complete with stainless steel sensor tube and T fitting, coupling cord and power cord. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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